ENDOTOXIN AS A NATURALLY OCCURRING IMMUNOMODULATOR

ENDOTOXINE ALS EEN NATUURLIJKE MODULATOR OP DE IMMUUNREACTIE

(*met een samenvatting in het Nederlands*)

PROEFSCHRIFT

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STELLINGEN

1

The search for the mot juste is not a pedantic fad but a vital necessity. Words are our precision tools. Imprecision engenders ambiguity and hours are wasted in removing verbal misunderstandings before the argument of substance can begin.

Anonymous (British) civil servant.

2

It is essential to know the extent to which Limulus positivity predicts the presence of other endotoxic properties and vice versa.

3

The immunological changes which attend surgery are not predictors of post-operative sepsis.

4

The relatively minor nature of many age-related changes may belie their true significance within a complex, integrated system.

5

Old age is not an immunodeficiency state.

6

Fever is an important host defence response and the use of antipyretic drugs may be detrimental to patients.

7

The existence of a specific process of ageing does not make sense in evolutionary terms.

8

In the main, experimental gerontology has neglected the major health issues of old age.

9

Medical technology has contributed but little to the public health.

10

In large organisations involved in scientific research it should be appreciated that scientific leadership is more than simply a matter of economic management.

11

Dutch architecture is inimical to the development of a progressive approach to the health care of the elderly.

12

The art of medicine is of at least equal importance to the science.

Stellingen behorende bij het proefschrift "Endotoxin as a naturally occurring immunomodulator."

M.A. Horan
May 20, 1986

The work described in this thesis has been performed at the
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CHAPTER I

NATURE AND ACTIVITIES OF ENDOTOXINS

1.1 INTRODUCTION

1.1.1 General Introduction

> "Endotoxins possess an intrinsic fascination that is nothing less than fabulous. They seem to have been endowed by nature with virtues and vices in the exact and glamorous proportions needed to render them irresistable to any investigator who comes to know them. The molecular basis for their biological action seems always on the verge of discovery but somehow just eludes detection".
>
> Bennett, 1963

Endotoxins are high molecular weight complexes which form an integral part of the gram negative cell wall. 130 years of research has demonstrated a wide variety of biological effects for these substances. Of equal interest is the variation in responses among higher animals to them e.g. the baboon is 10^4-10^5 times less sensitive to endotoxic effects than is man (see Westphal, 1984). This variation in response spreads right accross the animal kingdom. This led Menkin to propose that endotoxic effects were indirect; they were brought about by host mediator systems (see Westphal, 1984). Endotoxins are ubiquitous and no host can elude contact with them. Indeed, it has been calculated that some 4×10^5 ng per day are generated in the human colon. This very ubiquity has led some investigators to propose that interactions with endotoxins are indeed important in the development and functioning of some host systems and refer to them as a kind of "exo-hormone". It is with this possibility that this monograph is primarily concerned.

It is important to consider some historical landmarks in endotoxin research since this allows us to place current notions in the correct context.

1.1.2 Discovery and Isolation of Endotoxins

The story begins in Copenhagen in the middle of the last century where Peter Ludwig Panum (a pathologist) was investigating the mechanisms through which contagious diseases exerted their characteristic pathophysiological effects. He worked with infusions of decomposing human tissues and faeces, in search of some common principle.

To quote from Panum (1856):

> "the putrid fluid material was boiled for eleven hours......then the material was filtered. I injected 32 cc of the filtrate into the vena jugularis externa of a dog. Very soon afterwards, the dog showed weakness, increased pulse, nausea, chills, the conjunctivae were injected, pupilla were dilated; the dog increasingly lay down, showed signs of intense collapse, the pulse was extremely weak and unmeasurable. Lack of food and water intake. There is no doubt that this complex of symptoms is the same as that observed by me following injection of the original, unboiled, putrid fluid".

He demonstrated that after distillation, the toxic principle (putride gift) was in the residue and could be re-dissolved in water but not in alcohol. He established a lethal dose for the dog of about 0.12 grammes and that sub-lethal doses induced fever. He was convinced that living organisms could not have survived his extraction procedures and therefore proposed his "Chemical Theory of Intoxication and Septic Disease".

Around the time that Panum was working in Denmark, other researchers were investigating pyrogens. Billroth (1865) showed that distilled water could induce an elevation in body temperature, but only when contaminated with living or killed bacteria. Burdon-Sanderson (1876) called this fever-inducing product "pyrogen". This term remains in widespread use and fever production in the rabbit is still used as a means of detecting endotoxic activity. Roussy (1889) and Centanni (1894) tried to extract this substance by the fractionation of bacterial components. They showed that it was possible to sterilise culture supernatants by filtration, but that this procedure did not abolish pyrogen activity. This gave rise to the notion that bacterial lysis could release pyrogens into a cell-free supernatant. In 1892, Richard Pfeiffer coined the term "endotoxin" and defined it as "a poison which makes up part of the living substance of bacteria and is released only after their disintegration". We now know that endotoxins can be liberated by living bacteria under appropriate conditions (Russel, 1976).

Since these early days, numerous procedures have been described to extract bacterial endotoxins. Major contributions are summarized in Tables 1.1a and 1.1b (which are by no means complete). Most of the endotoxin preparations in current use are prepared by either the Boivin or Westphal method. No single procedure is applicable to all bacterial species and strains and the most valuable procedure must be individually determined. This difficulty is best illustrated by considering rough mutant bacterial strains. These strains have defects in the synthesis of the long polysaccharide side-chains and therefore possess very different polarities from native endotoxins. The term 'rough' merely refers to the colonial morphology of these strains when grown in culture. The Boivin procedure is ineffective and that of Westphal gives very poor yields. New methods had to be devised such as the chloroform-methanol procedure (Kasai et al., 1970; Chen et al., 1973) or the phenol-chloroform-petroleum ether technique (Galanos et al., 1969).

1.1.3 The structure of Endotoxins

Analysis of extracted endotoxin preparations show considerable heterogeneity. Over 40 components of the lipid moiety could be observed on thin layer chromatography (Kasai, 1966). It soon became clear that endotoxic activities were functions of lipopolysaccharides which are always bound to a small amount of protein: - Lipopolysaccharide associated protein, (LAP). Lipopolysaccharides (LPS) comprise a long polysaccharide covalently bound to a lipid region (Figs.1.1, 1.2). Westphal referred to this lipid as lipid A to distinguish it from non-covalently bound lipid. The core polysaccharide was often identical for large groups of bacteria (R antigen) and contains the unusual deoxy-sugar, Keto deoxy-octulosinic acid (KDO) as well as heptose, several hexoses and phosphoryl-ethanolamine. The remainder of the polysaccharide (O antigen) is subject to considerable variation between species and strains and confers the serotype specificity. Oligosaccharides in the R and O regions act as receptors for a number of bacteriophages which contain enzymes capable of degrading these oligosaccharides (and so have proved useful tools in endotoxin research). Portions of the polysaccharide are also recognised by cells of the host (particularly cells of the macrophage lineage) and may be important in the "clearance" of endotoxins and whole bacteria (see later).

TABLE 1.1a PROCEDURES USED TO PREPARE ENDOTOXINS

Method	Comments	References
Cell-free culture media	Good yield requires bacterial lysis. Heavily contaminated with other bacterial products. Allowed identification of a major polysaccharide component.	Centanni, 1894 Shear, 1941 Ikawa et al., 1952
Heating, filtration and alcohol precipitation	Derived by bacterial lysis. Heavily contaminated preparations. Very potent.	Centanni, 1894 Roberts, 1949, 1966
Enzymes	Serine proteases were the main enzymes used. Necessitated prolonged incubations. When serial enzyme digestion is applied to whole organisms, extremely potent preparations result. May be combined with acid hydrolysis and other purification steps.	Douglas & Fleming, 1921 Raistrick & Topley, 1934 Freeman & Anderson, 1941 Lehrer, 1971 Lehrer & Nowotny, 1972
Acid hydrolysis		
- 0.5 N TCA in cold	Further purified by dialysis and alcohol precipitation. Contains no nucleic acid. Endotoxin structure well maintained. 5-10% protein content. Potent endotoxin preparations. Remains in widespread use.	Boivin et al., 1933
- 0.1 N Acetic acid	Cleaves polysaccharide (Freeman polysaccharide).	Freeman & Anderson, 1941
Detergents + alcohol precipitation	Not contaminated with nucleic acids. Heavily contaminated with protein. Very potent endotoxins.	Nowotny et al., 1963 Braun & Rehn, 1969 Wu & Heath, 1973

TABLE 1.1b PROCEDURES USED TO PREPARE ENDOTOXINS

Method	Comments	References
Diethylene glycol	Difficult technique of limited value. Requires further dialysis and alcohol precipitation.	Morgan & Partridge, 1940
Phenol/water	First introduced in 1939. Refined by Westphal in 1952 by using 45% phenol in water at 68°C. On cooling, phenol and water separate and endotoxin is present in the aqueous phase. Can be further purified by alcohol precipitation. Very low protein contamination. Still in widespread use.	Miles & Pirie, 1939 Westphal et al., 1952
Phenol/chloroform/ petroleum ether	Useful for R forms.	Galanos et al., 1969
Pyridine	Not applicable to all organisms. Difficult procedure.	Goebel, 1945
Dimethylsulphoxide	Extraction at 60°C gives much higher yields than phenol extraction. Preparations very potent.	Adams, 1967

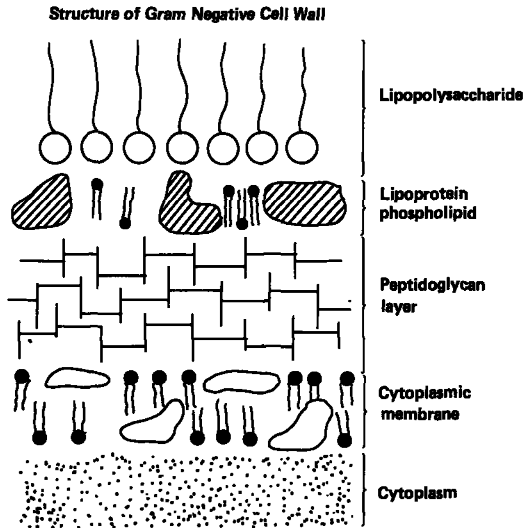

Fig.1.1 Structure of Gram Negative Cell Wall

The lipid A. is composed of β 1-6-linked D-glucosamine disaccharide units which carry a variety of amide and ester-linked fatty acids (including lauric, myristic, β-hydroxy myristic and palmitic) and pyrophosphate groups. Studies using chromatographically purified glycolipids from R mutant strains revealed that lipid(s) A was neither chemically nor biologically uniform. Most of the biological properties of endotoxins are concentrated in, but not necessarily confined to, the lipid A portion. It is therefore important to know, so far as possible, the chemical determinants of toxicity.

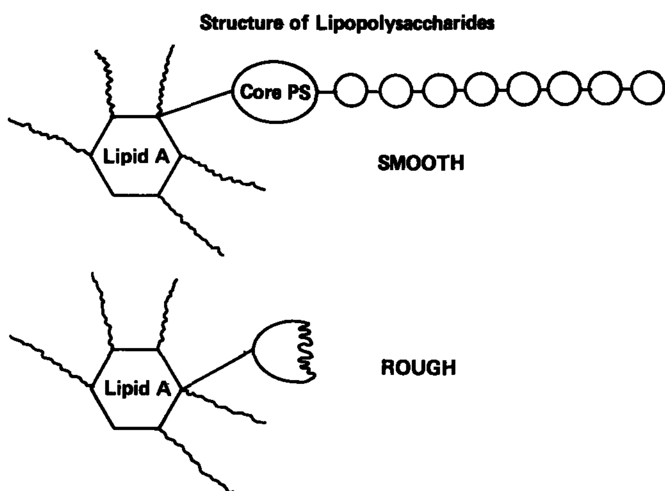

Fig.1.2 Structure of Lipopolysaccharides (for description, see text).

1.1.4 Structure - Function Associations

Studies using the Re 595 mutant of Salmonella minnesota demonstrated that the extracted LPS was fully active when compared to native endotoxin preparations (Kasai & Nowotny, 1967). Treatment with acid cleaves the polysaccharide from the lipid A which is then precipitated and can be removed. This lipid A also exhibits toxicity (Lüderitz et al., 1978). Mild alkaline hydrolysis removes ester linked fatty acids and results in a reduction of activity (Morrison & Ryan, 1979). Furthermore, the cationic antibiotic polymyxin B has been shown to bind stoichiometrically to the lipid A with a consequent loss of its biological activity.

Very recently, it has become possible to examine structure-function associations by chemically synthesising analogues of lipid A (Shiba et al., 1984). They tested thirteen acylated derivatives of β 1-6-D-glucosamine disaccharide with and without phosphate groups (based on the proposed structure of lipid A). They concluded that the amide-linked fatty acids and the 1-phosphate group were critical determinants of endotoxic effects. It was further demonstrated that the structural associations of toxicity were not uniform in that some properties were prominently displayed by some analogues while other properties were not (Matsura et al., 1984;

Tanamoto et al., 1984). For example, Limulus positivity was detected in only one analogue while mitogenicity and polyclonal B cell activation were found in most analogues. These results suggest that there may be multiple active sites in the lipid A molecule.

It seems likely that the polysaccharide component of LPS can modulate the expression of the properties of lipid(s) A. Studies with a Pseudomonas aeruginosa endotoxin preparation demonstrated that free lipid A could mediate pyrogenicity, adjuvanticity and interferon induction but could not inhibit experimental tumour development or induce clotting of the blood of the horseshoe crab, Tachypleus tridentus (Homma & Tanomoto, 1984). If lipid A is linked to KDO, full activity is seen, even though KDO has no intrinsic activity in either of these systems. Similar dissociations in activity have been described for Klebsiella lipopolysaccharides. Klebsiella LPS is a much more potent immuno-adjuvant than is E.coli LPS (Nakashima et al., 1984). Nowotny (1983) used a variety of "de-toxified" endotoxins to demonstrate that it is possible to retain potentially beneficial effects (e.g. protection against irradiation, mitogenicity, adjuvanticity and induction of endotoxin tolerance) while "toxic" (unwanted) effects were considerably attenuated.

In conclusion, it is clear that the various properties of endotoxin can be dissociated, both in synthetic analogues and in chemically modified extracted endotoxins. Although not directly demonstrated, the simplest explanation is that there are multiple active sites on the lipid A molecule which may be further modulated by the polysaccharide component, and possibly also by lipid A-associated protein (LAP). Furthermore, there is strong circumstantial evidence that the host bears receptors through which at least some endotoxin effects seem to be mediated (Section 1.2.4). The binding characteristics and distribution of such receptors might be expected to modify host responses to endotoxins.

1.2 THE IMMUNE SYSTEM AND ENDOTOXINS

1.2.1 Immune Response to Endotoxins

Deaths from gram negative septicaemia have decreased only slightly with the advent of potent antimicrobial chemotherapeutic agents and sophisticated life support systems. This has prompted intensive research into possibilities for immunological interventions (both prophylactic and therapeutic) in this setting. It is therefore of critical importance to understand the particular immunodeterminants responsible for antibody-mediated protection against whole organisms and LPS. There is no doubt that antibodies can be protective but no study has demonstrated protection by antibodies to one region of the LPS while excluding the presence of antibodies to other parts of the molecule.

Despite the chemical complexity of endotoxins, antibody responses are largely directed towards the O antigen (Morrison & Ryan, 1974). It is well known that sera

from normal individuals contain a variety of antibodies to O antigens. Purified LPS preparations are very potent immunogens and as few as 1000 "molecules" can elicit a primary antibody response when injected into a rabbit popliteal lymph node while even less can sensitise an animal for a subsequent secondary response. Rudbach (1971) refers to them as "super antigens" though it must be remembered that responses between different inbred strains of rats and mice may differ markedly. Chemical modification of the polysaccharide abrogates this specific antibody response (Neter et al., 1956). Injection of purified polysaccharide produced comparable responses in rats and mice, but not in rabbits or guinea pigs. This led to the suggestion that either lipid A or possibly LAP is an important modulator of the response to O antigenic determinants. Of interest in this regard is the observation that C3H/HeJ mice (which are refractory to lipid A effects) produce diminished O antigen antibody responses following injection of complete LPS.

Numerous studies have shown that antisera directed predominantly against O antigens protect against experimental infections with homologous bacterial strains, though the protection is highly specific, no protection being observed against other gram negative strains. Svenson (1979) demonstrated that both active and passive immunisation protected against murine typhoid, adding further support to the notion that antibodies directed against O antigens are protective.

A number of studies have shown that antibodies to the core polysaccharide can also provide protection in experimental infections (Braude et al., 1977; Bruins et al., 1977; Davis et al., 1978; Johns et al., 1977; Johns et al., 1983; McCabe et al., 1977; Ziegler et al., 1973). Antibodies to this region are cross reactive with a wide variety of gram negative bacteria. It will be remembered that this region of the molecule is structurally very conservative. Recently, Nelles and Niswander (1984) have produced monoclonal antibodies to core determinants of the LPS of the J5 mutant of E.coli O111:B4. They demonstrated cross reactivity with a wide variety of gram negative bacteria and that these antibodies did not bind lipid A directly. Binding to the core was inhibited by Polymyxin B and also by the presence of an O antigen. Human antibodies to the core polysaccharide have been obtained by immunising volunteers with the J5 mutant of E.coli O111:B4 and passive immunisation of patients with gram negative septicaemia was reported to reduce mortality by over 50% (Ziegler et al., 1982). The antibodies appear to function as an anti-toxin and give results far superior to using chemotherapeutic agents alone. The new human-human hybridoma monoclonal antibody technology promises new therapeutic tools for the prophylaxis and treatment at serious gram negative infections in humans (Teng et al., 1985).

Some authors have suggested that exposure to intact LPS does not induce antibodies to lipid A. This, however, seems not to be the case. Nolan has demonstrated that antibodies directed against lipid A are widely distributed among normal people (J. Nolan, 1985, personal communication).

1.2.2 Cellular Basis of the Anti-Endotoxin Response

Perhaps the most important work in this area was performed in the early 1970's when it was shown that the immune response to endotoxins could take place in the apparent absence of T cells (Andersson & Blomgren, 1971). Adult thymectomised, lethally irradiated, bone marrow reconstituted mice elaborated normal anti-endotoxin responses while responses to T cell dependent antigens (sheep red blood cells) was markedly reduced. Endotoxins were therefore referred to as T cell independent antigens. This has been confirmed many times since. In vitro treatment of spleen cells with anti-Thy-1 and complement prior to culture, suppresses the response to sheep red blood cells (SRBC's) but does not influence the anti-endotoxin response (Sjöberg et al., 1972). Indeed, the anti-endotoxin response is somewhat enhanced. Similar results were also reported in congenitally athymic (nu/nu) mice for both primary (IgM) and secondary responses (IgM + IgG) (see Morrison & Ryan, 1979). Looking at the question in reverse, the failure of activated T lymphocytes to influence the anti-endotoxin response further supported the T-independence of these antigens (Poe & Michael, 1976). However, other evidence exists to suggest that T cells can (and do) inhibit responses to endotoxins as do macrophages (to be summarised in 1.2.7 and 1.3.3). Nude mice do contain immature T cells and there is evidence that endotoxin can induce *maturation of these cells (Ikehara et al., 1984).*

It is generally accepted that at least three signals are required in T-cell dependent responses and recent work suggests that three signals are also required for LPS mediated T-independent responses (Zubler & Glasebrook, 1982). They used purified surface immunoglobulin positive murine lymphocytes (B lymphocytes) cultured at low density to minimise possible effects from contaminating T lymphocytes. They showed that LPS is able to by-pass the first step in which specifically activated T helper cells interact with B lymphocytes in the context of major histocompatibility antigens, but that antigen and non-specific T helper cell derived B helper factors are still required. When the antibody responses to LPS are considered, it follows that the LPS is also the antigen.

1.2.3 B Lymphocyte Activation

It is clear from Section 1.2.2 that endotoxin can interact with B lymphocytes in an antigen independent manner and this is the basis of some other interesting effects of endotoxins. The first experiments into endotoxin-induced B lymphocyte proliferation were reported by Takano et al. (see Morrison & Ryan, 1979). They followed incorporation of ^{32}P-labeled bases into the nucleic acid of spleen cells within 24-48 hours of an intraperitoneal injection of endotoxin. Their studies suggested that lymphocyte proliferation was occurring, probably in two sub-populations of cells with division times of 20 hours (lymphoblasts) and three days (lymphocytes) respectively. They suggested that endotoxin induced the differentiation of the former population into the latter. This report was supported by in

vitro work on murine splenocytes (Peavy et al., 1970). The T cell independence of this lymphocyte activation is supported by the lack of effect of pre-treatment with anti-Thy-1 and complement and its presence in congenitally athymic (nu/nu) mice.

Similar experiments were carried out in a number of animal species which broadly confirmed these results. However, there were occasional reports of minimal or absent responses, though these were explained away as differences in technique and in endotoxin preparations. One major stumbling block was that human peripheral blood lymphocytes could not be induced to proliferate with a wide range of endotoxin doses and with the presence or absence of human serum (Peavy et al., 1970). More recent studies (Miller et al., 1978) documented substantial proliferative responses in human spleen and abdominal lymph node cells, with lesser but definite responses in peripheral blood mononuclear cells, in the presence of fresh frozen pooled human serum and prolonged incubations (5-9 days); a result in keeping with our own findings (data not shown).

Endotoxin can not only induce B lymphocyte proliferation but also differentiation and antibody production. The first report that endotoxin could induce unrelated antibody synthesis was published by Ortiz-Ortiz and Jaroslow (1970). They demonstrated that in the absence of antigen, endotoxin induced a significant number of plaque-forming cells (PFC) to sheep erythrocytes and this is true for a wide variety of LPS preparations including purified lipid A. (Rank et al., 1972). Furthermore, PFC responses were demonstrated to erythrocytes of a wide range of animal species (but not mice - the experimental animal). This phenomenon has since been confirmed in a number of species, including man. Since then, antibody production to a wide range of determinants has been described (Table 1.2).

The induction of auto-antibodies is of particular importance to later discussions. It is also interesting that circulating immune complexes often accompany endotoxin-induced polyclonal activation, though the composition of such complexes is unknown. Two considerations should be borne in mind for later discussion. Firstly, the antibody may combine with the relevant antigen - if present. Secondly, it is highly likely that the host recognises the idiotypic determinants on these induced antibodies and mounts an immune response against them (anti-idiotypes). Furthermore, one might argue from network theory that some of the auto-antibodies may arise as a host response. Network theory (Jerne, 1974) predicts that as the antigenic determinant is unique, so is the conformation of the relevant combining site. It follows that this will be recognised by the immune system and induce the synthesis of a relevant antibody. The combining site of this second antibody must be structurally very similar to the original antigen (thus tending to limit the network rather than expand it). From the properties of endotoxins discussed above, it is possible that they induce anti-anti-idiotypes (which must be structurally very similar to the absent or hidden antigen) and the host may mount a response to this, leading to "auto-antibody" production.

TABLE 1.2 SPECIFICITY OF SOME ENDOTOXIN INDUCED ANTIBODIES

Specificity		Reference
Haptens:	*trinitrophenol* (TNP)	Izui et al., 1977
	dinitrophenol (DNP)	Izui et al., 1977
	phosphorylcholine (PC)	Louis & Lambert, 1979
		Rose et al., 1982
Soluble and particulate antigens		Izui et al., 1977
		Dresser & Popham, 1976
		Dresser, 1978
Autoantibodies		Dresser, 1978
		Fournié et al., 1974
		Hammarström et al., 1976
		Primi et al., 1977
Anti-idiotypes		Rose et al., 1982

Under normal circumstances, endotoxin can only interact with about 30-50% of murine splenic lymphocytes, but when combined with dextran sulphate (DxS), almost all the B cells present can be induced to proliferate (Bergstedt-Lindqvist et al., 1982). Furthermore, the need for accessory cells is abrogated (Wetzel & Kettman, 1981). It appears that endotoxin is most effective in triggering late G_1 cells into the S phase of the cell cycle.

1.2.4 The Nature of the Endotoxin-B Lymphocyte Interaction

Several models have been proposed to explain the nature of this interaction (Reviewed by Morrison & Rudbach, 1981). The "specific receptor" hypothesis is currently the most favoured. If this hypothesis is correct, one would predict that studies of LPS binding would reveal that it behaves in a similar way to other ligand-receptor interactions.

The binding characteristics of LPS to lymphocyte membranes has been extensively investigated in the mouse (Jacobs, 1982; Jacobs et al., 1983). They used a preparation from E.coli O55:B5 purified by the Westphal method. The LPS was detected using haptenated antibodies to the O antigen together with rhodamine-coupled antibodies to the hapten. They demonstrated that both specific and non-specific binding occurs, the former being most easily demonstrated with moderately low doses of LPS (10-50 µg/ml). Specific binding was competitively inhibited by another LPS (which was not recognised by the antibody) and was saturable, both with respect to dose and time. They then went on to investigate the nature of the

cells which bind LPS. In the spleen, around 50% of the cells bound LPS, but only about 15% of lymph node cells and 7% of cells in Peyer's patches. This binding pattern correlates directly with LPS induced mitogen responses by cells from these tissues. The majority of LPS-binding cells were μ^+, d^+, Ia^+, though a significant (albeit small) proportion of Thy-1$^+$ cells also bound LPS. It therefore appears that LPS binds predominantly to mature B lymphocytes and not to immature cells. This was confirmed by investigations into LPS binding by cells from neonatal mouse spleens.

It therefore appears to be true that B lymphocytes do bear receptors for LPS, the functional significance of which is unknown. It seems likely that it is polysaccharide that is recognised, but it is the lipid A that is responsible for most of the biological effects. Two other pieces of information cast doubt on the importance of membrane receptor binding in lymphocyte activation. Firstly, B lymphocytes from the C3H/HeJ mouse (which is genetically unresponsive to lipid A effects) bind LPS quite as well as B lymphocytes from other strains (Kabir & Rosenstreich, 1977). Secondly, LPS must be internalised before it can elicit B lymphocyte activation (Adler et al., 1972; Smith, 1972). This led McGhee and co-workers to postulate the presence of a cytoplasmic receptor for LPS, the binding of which is necessary to induce characteristic effects (Gollahon et al., 1983). In order to fully appreciate this concept, it is necessary to have some understanding of the genetics of LPS responsiveness in the mouse.

1.2.5 The Genetics of LPS Responsiveness

Our current understanding of this topic was initiated by the report by Hill and co-workers in 1940 that the inbred mouse strain C3H/HeJ was refractory to LPS-induced toxicity. Sultzer (1968) demonstrated that this unresponsiveness was genetically determined. Watson and co-workers (1977, 1978) showed that a single autosomal gene on chromosome 4 was responsible, the so-called LPS gene. The product of this gene has not been determined. The compatible strain C3H/HeN is fully responsive (LPS^n/LPS^n) while the C3H/HeJ mice are unresponsive (LPS^d/LPS^d). F_1 hybrids exhibit intermediate responsiveness (LPS^n/LPS^d). This is illustrated schematically in Fig. 1.3. The LPS gene maps near to genes for Lyb2, Lyb4 and Lyb6 and in the C3H/HeJ mouse, Lyb4 fails to map between Lyb2 and Lyb6. It seems a reasonable assumption that the LPS gene product determines the susceptibility or otherwise to LPS effects, though it is not known whether the defect at the LPS locus induces lack of, or excessive production of the gene product.

1.2.6 The Possibility of an Intracellular Receptor

This possibility was first put forward by McGhee and co-workers (Gollahon et al., 1983). There are precedents in other fields for such a possibility. Steroid hormones exert their effects at the cellular level by a similar mechanism. Cells sensitive to the relevant hormone bind the hormone and form an intracellular com-

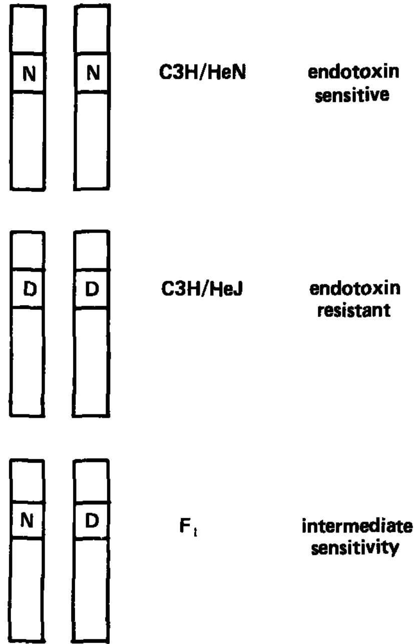

Fig.1.3 The genetics of LPS responsiveness in C3H mice.
Diagrammatic representation of the LPS gene on chromosome 4.

plex. The complex is then translocated to the nucleus where it interacts with DNA with subsequent expression of the typical effects of the hormone. It has already been explained in Section 1.2.4 that binding of LPS to the cell surface is not sufficient for the implementation of typical LPS effects. It has been shown that LPS induces phosphorylation of non-histone chromosomal proteins (reviewed by Decker & Marchalonis, 1978). This has been demonstrated in B lymphocytes within two hours of LPS stimulation (Stott & Williamson, 1978). It was therefore postulated than an interaction between LPS and the LPS gene product leads to transcription of DNA segments and ultimately cell division and maturation.

1.2.7 Interaction with T cells

It was reported in Section 1.2.4 that endotoxin binds a small number of lymphocytes which possess surface markers characteristic of T lymphocytes. LPS certainly enhances mitogen responses to phytohaemagglutinin and concanavlin A (Morrison & Ryan, 1979). It will also enhance the mixed lymphocyte reaction, even at doses which cannot be detected by currently available assays (Spear & Teodorescu, 1985). Recent studies have demonstrated that LPS can induce T cell proliferation, though this is not easy to demonstrate using conventional culture techniques (Mita et al., 1982). These responses could even be demonstrated in interleukin 2-dependent T cell lines in the absence of accessory cells (Vogel et al., 1983) and this effect could be abrogated by polymyxin B. Further aspects of LPS effects on T cell are discussed in Sections 1.2.8 and 1.2.9.

1.2.8 Endotoxin as an Adjuvant

It has been known for over 20 years that LPS could enhance the specific antibody response to exogenous antigens in vivo. Time relationships are critical for these effects and under certain circumstances, LPS can suppress responses. A plethora of in vitro studies have been reported which underline the complexity of this effect, and no unitary scheme can be proposed at present.

1.2.8.1 Thymus-dependent antigens

Spleen cells from congenitally athymic (nu/nu) mice, or from adult thymectomised, lethally irradiated, bone marrow re-constituted mice cannot respond to thymus-dependent antigens, but can do so in the presence of LPS (Sjöberg et al., 1972; Watson et al., 1973). Furthermore, LPS can readily reverse tolerance , even in the presence of antigen specific suppressor T cells (Skidmore et al., 1976).

A number of studies have been published which suggest that T cells are important in the exhibition of the adjuvant properties of LPS (reviewed in Nakano & Uchiyama, 1983). For example, Milner et al. (1983) have shown that LPS can facilitate "priming" of T cells using ovalbumin as antigen. Using in vitro techniques with purified T cells, B cells and macrophages from LPS-sensitive (C3H/HeN) and LPS-resistant (C3H/HeJ) mice, it has been shown that an adjuvant effect can be induced in B cells from lipid A-resistant mice provided sensitive T lymphocytes and macrophages are present (McGhee et al., 1979). Jacobs (1979) has shown that culture supernatants from activated T cells and macrophages can support the LPS-induced adjuvant effect on the response to erythrocyte antigens in macrophage-depleted spleen cell cultures under circumstances where LPS alone produced negligible effects.

1.2.8.2 T-independent antigens (TI antigens)(reviewed by Jirillo et al., 1984)

These are antigens which can elicit an antibody response in the absence of

mature T cells. However, some TI antigens appear to be "more independent" than others. On the basis of responses in the CBA/N-defective mouse, they can be classified as TI-1 and TI-2 antigens. These mice produce diminished responses to TI-1 antigens (such as LPS and LPS-conjugates) and almost no response to TI-2 antigens (such as TNP-Ficoll). There is evidence that there are separate sub-sets of B cells which respond to thymus-dependent (TD) and thymus-independent antigens. When the haptens DNP and TNP are conjugated to LPS, strong TI-1 responses are obtained. Endotoxin may also act as an adjuvant when co-administered with TI-2 antigens.

1.2.8.3 LPS-induced immune suppression

Augmentation of a specific antibody response has been discussed above. Under appropriate conditions, LPS can also suppress such a response. Behling and Nowotny (1980) have described this effect and determined that it is very time-dependent. Oscillating periods of enhancement and suppression occur depending on the time interval between administration of LPS and administration of the antigenic challenge. In vitro experiments have shown that not only is time an important factor but also that the animal strain used and the cell density in culture are important (Koenig & Hoffmann, 1979). Persson (1977) has shown that spleen cells from endotoxin treated mice could induce suppression of primary antibody responses in untreated cells, even when T cells and macrophages had been removed. Koenig & Hoffmann identified the cell type responsible as B lymphocytes.

It has already been mentioned that under appropriate circumstances, endotoxins induce suppressor T cells. This may play a regulatory function in vivo and is discussed further in Section 1.2.9. LPS can also induce immunosuppression by impairing the function of T helper cells (Portnoi et al., 1981). Macrophages too can mediate suppression. Support for this comes from the observation that the adjuvant activity of LPS in certain circumstances is diminished when spleen cell cultures are depleted of macrophages (Nakano & Uchiyama, 1983).

1.2.9 Endotoxin Regulation of Mucosal Immunity

Sections 1.2.1-1.2.8 have reviewed LPS interactions with the immune system. The question remains whether these interactions are of any relevance outside the experimental situation. The normal enteric flora generates considerable quantities of LPS (in man, up to 400,000 ng/day) and one might anticipate that gut-derived LPS exerts an effect on gut associated lymphoid tissue. Recent work has demonstrated that this is indeed the case. (Kiyono et al., 1982; Mickalek et al., 1983; Wannemuehler et al., 1982; Williamson et al., 1984).

Germfree (GF) mice immunised orally with the TI-1 antigen TNP-LPS showed good splenic PFC responses in the absence of added T cells. Addition of T cells

from conventionally-maintained mice of the same strain suppressed this response. T cells from GF mice had no effect. Comparing responses between GF and conventional animals revealed that GF animals always exhibited higher responses. GF mice returned to the conventional state exhibit identical responses to animals which have always been maintained under conventional conditions.

Prolonged oral administration of the TD antigen, sheep red blood cells (SRBC) results in unresponsiveness to a parenteral challenge with the same antigen (oral tolerance). If the hypothesis that gut derived LPS induces suppressor T cells is correct, one would predict that the LPS unresponsive mouse (C3H/HeJ) would not develop oral tolerance. They demonstrated that this was indeed so. The same phenomenon was demonstrated for G.F. mice compared to conventional mice. It is interesting that the Peyer's patches of C3H/HeJ mice primed with SRBC's contained considerable T helper activity and little suppressor activity while those from C3H/HeN mice showed just the opposite.

This is strong circumstantial evidence that gut-derived LPS affects the gut associated lymphoid tissue in LPS responsive animals. It has been clearly demonstrated that this interaction generates an easily detectable suppressor T cell population. Whether these T cells arise as a direct result of an LPS-T-cell interaction or arise in response to B cell activation (or a combination of the two!) is not known.

1.3 INTERACTIONS WITH MACROPHAGES

1.3.1 Introduction

Following Menkin's suggestion in 1956, it is now widely believed that a great many endotoxic effects are indirect. The evidence is burgeoning that cells of the mononuclear phagocyte system are responsible for the elaboration of the mediators of endotoxic effects. It is also widely believed that not only is the mononuclear phagocyte system responsible for the production of mediators, but is also responsible for the removal of endotoxins from the body and their subsequent destruction. This latter topic will be covered in detail in Chapter VII.

1.3.2 Mononuclear Phagocytes

The story of phagocytic cells began just over a century ago when the Russian zoologist, Elie Metchnikof, was taking a holiday at Messina on the Mediterranean seashore. He was studying amoeboid cells in starfish larvae. "The Sicilian seashore provided a ready supply of starfish and, one might surmise, the correct ambience for a 'gentleman scientist' with an aversion to Russian winters".

On the day in question, in December 1882, Metchnikof was seized with an inspiration:

> "It struck me that similar cells might serve in the defence of the organism against intruders. Feeling that

this was something of surpassing interest, I felt so exited that I began striding up and down, and even went to the seashore to collect my thoughts. I said to myself that, if my supposition were true, a splinter introduced into the body of a starfish larva should soon be surrounded by mobile cells, as is observed in a man who runs a splinter into his finger."

He went on to demonstrate that this was indeed the case and referred to these cells as phagocytes*. The significance of this concept was soon recognised and Metchnikof was awarded the Nobel Prize for Medicine (jointly with Paul Ehrlich) in 1908. Metchnikof referred to polymorphonuclear leucocytes as microphagocytes and macrophages as macrophagocytes.

Macrophages derive from a bone marrow precursor which differentiates into a monoblast, then promonocyte, then monocyte. Monocytes are released into the circulation but are still immature cells. They migrate within hours to days into various tissue destinations where they can exist for very long periods. In the lung, they become alveolar macrophages; in the spleen and peritoneal cavity they become tissue macrophages; in the liver they become Kupffer cells (though Kupffer cells are certainly capable of replication in situ) and in bone they become osteoclasts.

Following Metchnikof's lead, phagocytosis was the most investigated property of this mononuclear phagocyte system. Indeed, their capacity for phagocytosis is nothing short of awesome in that they can ingest more than 50% of their own mass of particulate matter. For example, it has been calculated that in the course of a year, cells of this population ingest and digest more than 2 Kg of haemoglobin from effete red blood cells.

In recent years it has become clear that cells of the mononuclear phagocyte system may participate in wound healing, remodelling of tissues and in the development of atheromas. Furthermore, macrophages are also secretory cells and a summary of their secretory products is given in Table 1.3 (Nathan et al., 1980). The precise regulation of the production of these products in vivo remains unclear. Recent studies on human peripheral blood moncytes have suggested that different sub-populations with different properties exist (Khansari et al., 1985) and these are summarised in Table 1.4.

* phagein = to eat
kytos = hollow vessel

TABLE 1.3 SECRETARY PRODUCTS OF MONONUCLEAR PHAGOCYTES

Enzymes	Lysozyme		
	Neutral proteases:	Plasminogen activator	
		Collagenase	
		Elastase	
		Angiotensin converting enzyme	
	Acid hydrolases:	Proteases	
		Lipases	
		Ribonucleases	
		Phosphatases	
		Glycosidases	
	Arginase	Sulphatases	
Complement Components	C1	Reactive Oxygen Metabolites	Superoxide
	C4		Hydrogen peroxide
	C2		Hydroxyl radical
	C3	Bioactive Lipids	Prostaglandins
	C4		Thromboxane
			Leukotrienes
	Factor B	Monokines	Interleukin 1
	Factor D		Colony stimulating factor
	Properdin		Interferons
	C3b inactivator		Tumour necrosis factor
Enzyme inhibitors	Plasmin inhibitors		Glucocorticoid antagonistic factor
	α_2 macroglobulin		
Binding proteins	Transferrin		Insulin-like activity
	Transcobalamin II		
	Fibronectin		

TABLE 1.4 SUBPOPULATIONS OF HUMAN MONOCYTES

Population	% Cells	IL-1 production	PGE_2 production	Accessory cells for Ig production
A	7	+	+	+
B	11	+	+	+
C	28	±	+++	suppress.
D	34	+++	±	+++

1.3.3 Endotoxin - *Macrophage Interaction*

Endotoxin binding to macrophage membranes in vitro is well established (Silver, 1981) *and this binding is followed by internalisation.* Studies with tritium labeled LPS from Bordetella pertussis showed that this binding was saturable and could be inhibited when peritoneal macrophages were under study, but binding could not be inhibited in alveolar macrophages, where binding was non-specific (Haeffner-*Cavaillon et al.,* 1982). Binding could be inhibited by a polysaccharide present in the endotoxin (PS-1) but not by lipid A. Membrane binding in the C3H/HeJ mouse is unimpaired and it is has been suggested that an intracellular receptor mediates endotoxic effects on macrophages (Gollahon et al., 1983). Undoubtedly, endotoxin can induce effects on isolated cells in vitro without any need for mediators and binding has been demonstrated to these cell membranes (Silver, 1981). The specificity of this binding remains to be determined.

The primary morphological changes in activated macrophages are reviewed by Morrison and Ryan (1979). The primary morphological change, enhanced spreading on surfaces, is not seen in the C3H/HeJ mouse following endotoxin injection. Numerous other morphological changes have been described which include increased membrane "ruffling" and changes in cytoplasmic granules. Concomitant with morphological changes is an enhancement of phagocytic function. This is associated with an increase in certain membrane *receptors such as* the receptor for C3. Depression of hepatic macrophage complement receptor function is associated with enhanced sensitivity to endotoxins (Loegering & Blumenstock, 1985). Macrophages are much more sensitive than lymphocytes to the action of LPS, nanogram amounts or less being effective. It is reported that sensitivity to activation is accompanied by an increased sensitivity to the cytotoxic effects of LPS. A concentration of 10 µg/ml of endotoxin in serum-free medium induced extensive cytotoxicity (as judged by failure to exclude trypan blue) within six hours. Other experiments have demonstrated that the effects of LPS *on murine peritoneal macrophages* were also very dependent upon LPS concentration, the presence of serum and the age of the

cultures. Low LPS concentrations, high serum concentrations and more differentiated cells tended to correlate well with stimulation as opposed to cytotoxicity. Results from this laboratory (Institute for Experimental Gerontology) have shown that isolated rat Kupffer cells are resistant to cytotoxicity from milligram concentrations (A. Brouwer, personal communication). Endotoxin inhibits the _in vitro_ migration of guinea pig peritoneal macrophages, and Marcol-elicited macrophages are much more sensitive to this effect than are normal (unstimulated) resident peritoneal macrophages (Fox & Rajaraman, 1980). Viability was unimpaired at the dosage used (1-10 µg/ml) and the effect could be readily reversed by polymyxin B. Nagao et al. (1984) reported that liquid paraffin-elicited guinea pig peritoneal macrophages displayed depressed DNA synthesis over controls following exposure to endotoxin (E.coli, Westphal method). The short time course of the effect suggested that it was not secondary to the production of mediators such as prostaglandin E_2.

Part of the sensitivity or resistance may also relate to the ability of macrophages to detoxify endotoxins. Filkins (1971) showed that incubation with liver, lung, spleen or peritoneal macrophages markedly reduced LPS-induced lethality in lead acetate treated mice. The precise mechanism of detoxicification is, as yet, unknown.

1.3.4 Endotoxin-Induced Production of Mediators

Table 1.3 summarises secretory products of macrophages, many of which can be induced by LPS. Most of these effects are not strictly germaine to the present discussion. Only arachidonic acid metabolites (eicosanoids) and interleukin(s) 1 will be considered, as these are of the most obvious immunological relevance.

1.3.4.1 Eicosanoids

Prostaglandins and leukotrienes are difficult to study since they are present only in low concentrations, are produced locally and are rapidly degraded. They are synthesised at or close to cell membranes from at least three closely related 20-carbon atom parent compounds. Arachidonic acid is the best known of these. Synthesis products are not stored but are formed immediately prior to their release. Arachidonic acid is formed by the action of phospholipase on phospholipids. It is possible that LPS may increase the supply of phospholipids by a direct effect upon membranes. It is known that glucocorticoids inhibit the mobilisation of phospholipids from membranes and protect against many endotoxic effects. The cyclo-oxygenase pathway (which gives rise to prostaglandins and thromboxanes) can be inhibited by non-steroidal anti-inflammatory drugs while the lipoxygenease pathway (which gives rise to leukotrienes) cannot, except at high concentration.

Since some endotoxic effects can be inhibited by cyclo-oxygenase inhibitors, a role for prostaglandins has been proposed in the mediation of endotoxic effects (Reviewed by Schade & Rietschel, 1982). Such effects include death, diarrhoea,

generalised Schwartzman reaction, inflammation of the eye, immobilisation of the guinea pig knee joint, fever, abortion in mice, accelerated ovum transport in rabbits and the early phase of shock. Since it has been demonstrated that macrophages are necessary for many of these effects, they were the first cells studied. It was shown that LPS could induce synthesis of PG E_2 and PG $F_2\alpha$ in vitro in murine macrophages derived from the bone marrow or peritoneum, in human peripheral blood monocytes, in rat Kupffer cells and in rabbit alveolar macrophages. Some of these cell types may exist as distinct sub-populations with different potentials for the production of prostaglandins (Section 1.3.2).

Lipid A is responsible for the induction of prostaglandins and chemically modified lipids A have a reduced capacity to do so. Furthermore, macrophages from the lipid A resistant C3H/HeJ mouse show minimal responses. In sensitive strains, prior administration of LPS markedly modifies the response to a subsequent LPS challenge. Injection of a sub-lethal dose of LPS 24 hours before a challenge produces enhancement while a challenge four days after the priming dose produced up to a five-fold increase in resistance. These states of sensitivity and resistance are associated with changes in prostaglandin synthesis (Schade & Rietschel, 1982). These effects are summarised in Table 1.5. This phenomenon is specifically restricted to LPS since stimulation of "tolerant" macrophages by zymosan results in normal production of both the prostaglandins studied.

The patterns of endotoxin production in vivo differ from the in vitro effects described above. Some of the differences may relate to species variation and prostaglandin degradation in vivo. Studies on experimental endotoxin shock

TABLE 1.5 PROSTAGLANDIN SYNTHESIS BY MURINE MACROPHAGES

condition of mice	PG E_2	PG $F_2\alpha$
untreated	7.0 ng/ml	0.02 ng/ml
sensitised	13.5 ng/ml	0.76 ng/ml
resistant	0.7 ng/ml	0.04 ng/ml

in baboons revealed a close correlation between endotoxic effects and the levels of thromboxane B_2 and prostacyclin (PG I_2). Furthermore, infusion of PG I_2 into dogs rendered them less sensitive to endotoxin. It was also demonstrated that murine peritoneal macrophages produced thomboxane B_2 and PG I_2 in response to zymosan but not in response to LPS. Leukotrienes are highly active mediators of inflammatory and hypersensitivity reactions and leukotriene C_4 (LTC_4) antagonists are able to prevent, to a large extent, endotoxic shock in D-galactosamine-

-sensitised mice. (Schade et al., 1984). Endotoxin not only induces the production of leukotrienes in vivo (rat as experimental animal) but also impairs their elimination (Hagmann et al., 1985).

Our understanding of precisely how these arachidonic acid metabolites are regulated in vivo remains fragmentary and no precise scheme can be constructed as to how they contribute to the pathophysiological changes following endotoxin administration.

1.3.4.2 Interleukin 1 (IL-1)

Interleukin 1 activity is a function of cells of the mononuclear phagocyte system and cerebral astrocytes (Fontana et al., 1984). It is widely assumed that leukocyte endogenous mediator (LEM), leukocyte activating (LAF) and endogenous pyrogen (EP) are one and the same substance, IL-1. Originally, the term IL-1 referred to a monokine of molecular weight 15,000, which induces secretion of T cell growth factor (interleukin 2) and stimulates B cells to differentiate. It is now known to occupy a central position in the acute phase response (APR), mediating fever, neutrophilia, and changes in liver and muscle metabolism (Dinarello, 1984). Its production is probably unimpaired in ageing (see chapter VII). It has recently been shown that human interleukin 1 activity is mediated by at least four closely related but biochemically distinct peptides (Wood et al., 1985), all of which display similar properties (but certainly differ in some of their properties). It has also been demonstrated that two biochemically and immunologically distinct forms of porcine interleukin I have identical biological properties (Saklatvala et al., 1985).

Numerous studies report IL-1 release by endotoxin stimulation of cells of the mononuclear phagocyte system (e.g. Gery & Waksman, 1972). Human monocytes are extremely sensitive to LPS with respect to IL-1 production. Maximal stimulation can be elicited by just 100 pg/ml (comparable to what might be encountered in vivo) (Lachman, 1983). Polymyxin B is able to abort this response, but only if given before the addition of serum to the culture. Prior incubation of LPS with serum prevents the inhibitory effect produced by polymyxin B and this effect has not been adequately explained. Binding to high density lipoprotein may be important (chapter VII).
Some authorities propose that the hepatic Kupffer cells are the main source of interleukin 1 in vivo (reviewed by Greisman 1983). For example, isolated rabbit Kupffer cells from animals rendered tolerant to endotoxin were found to be refractory to endotoxin-induced IL-1 production while alveolar macrophages showed normal responses. Furthermore portal vein endotoxin infusion in non-tolerant rabbits resulted in much higher fevers than did infusion into a systemic vein.

1.4 ENDOTOXIN AND PLASMA MEDIATOR SYSTEMS

This topic is well reviewed by Morrison and Ulevitch (1978) and only the major conclusions will be presented here.

1.4.1 Complement

It is well known that complement is consumed during experimental endotoxic shock and in gràm negative sepsis. This led to a series of experiments designed to elucidate the mechanism of LPS induced complement activation. Two antibody-independent mechanisms have been defined. Lipid A can bind C1 leading to activation of the classical pathway. The presence of polysaccharide, which activates complement by the alternative pathway also modifies lipid A binding to C1. LPS preparations from bacterial strains with defective polysaccharide synthesis show low or absent complement activating activity.

1.4.2 Coagulation System

LPS-induced activation of the clotting system was noted in the 1920's. Sanarelli and Shwartzman (working independently) demonstrated that two appropriately spaced doses of LPS could induce disseminated intravascular coagulation (DIC). It was later discovered that LPS could activate both the intrinsic and extrinsic systems. Activation of the extrinsic system is indirect and depends on a tissue factor released from activated endothelial or mononuclear cells. Activation of the intrinsic system depends on activation of factor XII (Hageman factor) by lipid A. This factor XII activating activity is enhanced by the presence of the polysaccharide, but polysaccharide is not esssential for this effect. Activated factor XII can also activate the kinin system with the generation of vasoactive substances. However, the presence of an intact intrinsic pathway, or indeed of platelets, is not essential for LPS-induced DIC.

1.4.3 Platelets

Numerous studies have demonstrated that LPS can exert profound effects upon platelets from various mammalian species (reviewed by Morrison & Ulevitch, 1978). As with most endotoxic effects, the effect on platelets shows considerable variation between species. Rat, rabbit, dog and guinea pig are much more sensitive than primates and this sensitivity is thought to depend on immune adherence sites on platelet membranes (which primates lack); more specifically, C3 receptors. The in vitro effect of LPS on platelets has been extensively investigated in rabbits and mice. (Morrison et al., 1981). They established that LPS could induce the platelet release reaction in a dose dependent fashion, but this was absolutely dependent on the presence of calcium. Electron microscopic studies showed that LPS induces morphological changes with the appearance of finger-like projections and pseudopodia. The presence of this morphological change conferred the ability of the platelets to respond to calcium. Since this response is intact in the C3H/HeJ mouse, it was concluded that it must be a non-specific property of lipid A binding to platelet membranes that is responsible.

1.5. METABOLIC ASPECTS OF ENDOTOXAEMIA (Spitzer 1983)

The administration of endotoxin has profound effects on the metabolism of an organism which are mainly catabolic. To what extent these effects are a direct endotoxin effect, are related to the elaboration of mediator substances or are due to changes in blood flow, is unclear.

Following endotoxin administration, there is a significant fall in cardiac output and mean arterial pressure. Provided the severity of the endotoxic insult was not too great, both cardiac output and mean arterial pressure tend to increase towards their initial levels. Nevertheless, while blood flow to vital organs such as liver and brain tends to be maintained, blood flow to other organs and tissues may be severely reduced. It is believed that hypoperfusion is responsible for a transient hyperinsulinamia followed by normal (or even depressed) plasma insulin levels together with sustained hyperglucagonaemia (Spitzer et al., 1980).

The first change in carbohydrate metabolism is a transient hyperglycaemia due to increased glycogenolysis in the liver (and possibly skeletal muscle). Gluconeogenesis is increased (using mainly alanine and lactate as substrates) but this fails to keep pace with the increased glucose utilisation of peripheral tissues. One possible explanation for this is that endotoxin prevents the induction of phospho-enol pyruvate carboxykinase (PEP-CK) by cortisol, and this is a rate limiting step in gluconeogenesis. This effect is mediated by the monokine, glucocorticoid antagonistic factor (GAF). The effect appears to be exerted at a stage before mRNA production since the levels of specific PEP-CK mRNA are reduced (Berry & Huff, 1984).

Free fatty acids are not utilised as an energy source since, although strong lipolytic signals are present, there is no net free fatty acid release due to severely impaired blood flow to adipose tissue.

There is net protein degradation in skeletal muscle at the same time as the liver markedly increases its production of some proteins (acute phase proteins) as well as decreasing production of others (e.g. albumin, transferrin). The relative contributions of monokines, changes in blood flow and direct endotoxic effects to this negative nitrogen balance are unknown (Filkins, 1985).

1.6 ENDOTOXINS AND THE LIVER

This topic is more properly the subject of Chapter VII. However, it is important to raise the notion that the liver is an import organ for LPS clearance and may also be a site of lymphocyte activation. For these reasons, a brief discussion of liver-endotoxin interactions will be presented here, though clearance and detoxicfication of LPS will be excluded.

1.6.1 Liver Excretory Function (reviewed by Utili et al., 1977)

Bernstein and Brown first described a syndrome in infants of cholestasis

associated with extra-hepatic gram negative infections. There are high levels of plasma bilirubin associated with bilirubinuria and the histological appearance of intrahepatic cholestasis with little evidence of parenchymal cell injury. Hirsch and co-workers demonstrated an increase in bromsulphthalein (BSP) retention within twenty minutes of endotoxin administration to rats. Studies on isolated perfused rat livers showed dose dependent decreases in perfusate flow, BSP excretion and bile flow (Abernathy et al., 1980). Controls perfused at the same flow rates as experimental animals did not show comparable falls in bile flow or BSP excretion. Analysis of BSP disappearance curves suggested that excretion was impaired. A similar phenomenon was observed for indocyanine green (which does not require conjugation for excretion). Other experiments showed that the fall in bile flow was predominantly related to bile salt-independent flow (which comprises about 35% of total bile flow; Erlinger et al., 1970). In infants, bile salt-independent flow is the major contributor to bile flow and hence, anything interfering with this might give rise to cholestatic jaundice in infants.

1.6.2 Vascular Effects

Studies in intact animals and isolated perfused livers from both dogs and rats have shown a very rapid obstruction to venous outflow from the liver with a resultant increase in pressure in the portal vein and hepatic artery (Utili et al., 1977). The effect is dose-dependent and is observed even when the liver is perfused entirely with synthetic medium. Antagonists of adrenergic and serotonin activity do not modify the response but glucocorticoids do. This hepatic pooling effect is not observed in primates and vaso-active substances are liberated in large amounts. The possibility therefore arises that endotoxin exerts its effects on flow through the rat and dog liver by a direct effect on sinusoidal lining cells. It has long been known that LPS produces widespread damage to vascular endothelium and endothelial cells can be found in the blood within five minutes of LPS injection (reviewed by Morrison & Ulevitch, 1978).

1.6.3 Endotoxin Induced Cytotoxicity

Using near lethal doses of LPS, marked changes can be seen in the liver within fifteen minutes of injection in a number of species (reviewed by Utili et al., 1977). The sinusoids appear congested, loss of glycogen occurs, vacuoles appear in the cytoplasm of hepatocytes and mitochondria are swollen. The space of Disse becomes dilated and Kupffer cells are swollen. By one hour, mitochondrial swelling is more marked and fibrin deposition can be seen in the micro-circulation. These changes worsen over the next few hours and can still be observed at 24 h post-injection. Disturbances in mitochondrial function accompany these changes which cannot be explained by hypoxia alone. Hepatocellular damage is inferred by release of enzymes, but it is not established that this is a direct effect of LPS.

1.6.4 In-vivo Responses of the Liver (McCuskey et al., 1982)

It has recently become feasible to follow events on the sinusoidal lining at the microscopic level and studies have been carried out aimed at defining Kupffer cell function in the living liver in relation to LPS administration. In these studies, Kupffer cell function was assessed by measuring the rate of phagocytosis of injected latex particles. In control NMRI mice, the mean time for phagocytosis of a single particle was 27 seconds. Administration of sublethal doses of endotoxin resulted in an increased rate of phagocytosis (by 33%) at 5 minutes following injection but by 15 minutes, phagocytosis was suppressed by about 37%. At this time, leucocytes (including lymphocytes) and platelets could be seen to transiently adhere to the sinusoidal lining. By two hours the velocity of blood flow was reduced and platelet and leucocyte plugs could be seen in association with swollen Kupffer and endothelial cells.

Mice rendered tolerant to endotoxin failed to show a reduction in the rate of phagocytosis following administration of an LD_{70} dose of LPS. Twenty four hours after administration of a tolerance-inducing dose, a modest enhancement of phagocytosis (12%) could be recorded. Studies in C3H/HeJ mice showed no change in the phagocytic rate, but the number of Kupffer cells that participate in phagocytosis was dramatically reduced while the total number of Kupffer cells appeared to be unchanged.

1.6.5 Kupffer Cells and the Immune Response

Kupffer cells are the representatives of the mononuclear phagocyte system (Section 1.3) which line the hepatic sinusoids (in association with a number of other cell types). They are believed to originate from circulating moncytes. Indeed, it has been calculated that up to 50% of circulating monocytes are destined to become Kupffer cells (Crofton et al., 1978) and survive there for some 3-16 weeks. Although the phagocytic capacity of Kupffer cells is nothing short of awesome, evidence has accrued which suggests that they also function as participants in immune responses.

The formation of porta-caval anastomoses (diverting some portal venous blood directly into the systemic circulation) abolishes the ability to induce oral tolerance (Section 1.2.9) to some antigens (Cantor & Dumont, 1967). Injection of some antigens directly into portal tributaries of normal guinea pigs induces immunological tolerance while systemic administration results in a normal immune response (Battisto & Miller, 1962). More recent studies in mice have shown that phagocytic blockade of Kupffer cells by colloidal carbon or dextran increases the antibody responses to sub-optimal doses of sheep erythrocytes some tenfold (Souhami, 1972). These studies do not prove a direct role for Kupffer cells in the generation of an immune response and could merely indicate that exposure to antigen is enhanced because of defective removal. In order to determine whether they can

function directly as participants in an immune response requires separation of Kupffer cells and using them for in vitro experiments.

Guinea pig Kupffer cells have been isolated for use in such in vitro experiments. The assay system used was to prime isolated Kupffer cells with antigen and then assess the proliferative response of T lymphocytes obtained from previously primed guinea pigs. Using this system, it was shown that Kupffer cells could indeed act as antigen presenting cells but were less efficient than elicited peritoneal macrophages (Rogoff & Lipsky, 1981). This difference could not be explained by defective uptake of antigen. Antigen presenting cells must display antigens in the context of the relevant surface histocompatibility antigens and not all cells of the mononuclear phagocytic system display these antigens. It is possible to kill Ia bearing cells by incubation with an anti-Ia antiserum and complement. Cells which do not bear Ia antigens survive this procedure, but do not function as antigen presenting cells. The isolated Kupffer cell population contained only 50% of the number of Ia positive cells seen in a comparable peritoneal macrophage preparation. The reasons why so many Kupffer cells fail to display Ia antigens are at present unknown.

Since some Kupffer cells do have the potential to act as antigen presenting cells, it is reasonable to assume that factors in their specific micro-environment may inhibit interaction with lymphocytes. It is therefore interesting that in experimental endotoxaemia, lymphocytes can be seen to adhere to the sinusoidal lining and perhaps interact with antigen presenting Kupffer cells.

1.6.6 Endotoxins and Liver Disease

The first evidence that endotoxins may contribute to liver injury and the characteristic pathophysiological changes associated with liver diseases comes from studies on experimental liver diseases in rats. For example, the hepatic necrosis associated with choline deficiency does not occur in germ free rats or in rats pre-treated orally with non-absorbable antibiotics (for review, see Nolan, 1981). Furthermore, addition of endotoxin to the drinking water of these rats completely negated the protective effect of the germ free state or oral antibiotic administration. It has subsequently been shown that endotoxins may contribute to liver injury associated with galactosamine, carbon tetrachloride and ethanol. Systemic endotoxaemia has been demonstrated (using the LAL gellation test) in all these experimental liver diseases, and in others (portacaval anastomosis, α-Naphthyl-isothiocyanate, common bile duct ligation). Endotoxins were detected systemically in small amounts in less than 5% of control animals.

The observations described above point to a role for gut-derived endotoxins in the pathogenesis of the liver injury. This raises the question of endotoxin absorption. One study using ^{51}Cr labeled endotoxin provided no evidence of absorption (Sanford & Noyes, 1958) while another study employing ^{32}P-labeled bacteria gave unequivocal evidence that endotoxins were indeed absorbed (Ravin et

al., 1960). Studies using isolated, everted gut sacs showed that labeled LPS passes transmurally in small amounts and that this seems to be associated with an active carrier mechanism rather than simple, passive diffusion (Nolan et al., 1977). Recently, doubt has been cast upon the use of everted gut sacs for studying endotoxin absorption (Ouwendijk, 1985). Using closed "in-situ" gut loops, this author was able to confirm endotoxin absorption, but in much smaller quantitites than are seen with everted gut sacs. Nevertheless, other studies confirm that endotoxin is a usual constituent of human portal venous blood (Jacob et al., 1977).

Although porta-systemic shunting of blood will undoubtedly contribute to the appearance of endotoxin in the systemic circulation, the notion has already been proposed that Kupffer cells play a critical role in endotoxin clearance and detoxification. Frog virus 3 fails to replicate in mammalian cells but is concentrated in Kupffer and endothelial cells (Gut et al., 1980). This is associated with total loss of Kupffer cells and severe damage to endothelial cells within hours of injection. Massive hepatic necrosis follows the damage to these sinusoidal lining cells. Challenge of colectomised rats with Frog 3 virus does not abort the damage to the sinusoidal lining cells, but does prevent the hepatic necrosis.

Endotoxaemia is associated with a variety of liver diseases in man including viral hepatitis, massive hepatic necrosis, cirrhosis, alcoholic hepatitis, chronic active hepatitis and obstructive jaundice (reviewed by Wilkinson, 1977). Severity of disease correlated crudely with the amount of endotoxin detected. Of special interest to the present discussion is the well known association of chronic liver diseases with immunological abnormalities, even in liver diseases which do not have an immunological aetiology. Such changes include polyclonal immunoglobulin elevations, circulating auto-antibodies and immune complexes. Such changes can all be induced by endotoxins (Chapter I). Unpublished results from our studies in Manchester on patients with obstructive jaundice demonstrated low levels of endotoxin in over 70% of patients, and that systemic endotoxaemia was associated with a marked increase in the proportion of DNA synthesising lymphocytes in the peripheral blood. Combining autoradiography with detection of surface antigens revealed that over 50% of the "spontaneously active" T lymphocytes expressed both the T4 <u>and</u> T8 antigen. It has recently been shown that such "co-expression" is a property of cells which have the morphology of lymphoblasts (Blue et al., 1985). It is therefore suggested that the endotoxaemia observed in a variety of liver diseases is associated with lymphocyte activation and the generalised immunological aberrations outlined above.

1.7 CONCLUDING REMARKS

The information presented in this chapter serves two major functions:

1. To describe the nature of endotoxins;
2. To describe the biological activities of endotoxins, especially as they <u>might</u> relate to the lympho-reticular system (and ageing).

Concerning the first aim, it has been emphasised that the biological properties of endotoxins are concentrated in the lipid moiety, though all portions of the molecule are immunogenic in their own right, under appropriate conditions. The combination of polysaccharide with lipid A may modify the biological properties of the latter. The role of lipid-A-associated protein (LAP) is much more speculative. This substance is endowed with biological activities in its own right and its presence may complicate studies on the mechanisms by which endotoxins exert their effects. Even the most stringent purification procedures result in products containing about 1% protein. The use of synthetic lipid(s) A analogues may serve to circumvent this problem and the results of more such studies are eagerly awaited.

With regard to the second aim, the interactions of LPS with the host lympho-reticular system have been outlined. These interactions are summarised in Table 1.6.

TABLE 1.6 IMMUNOLOGICAL EFFECTS OF LPS

1) B cell mitogenicity
2) Polyclonal antibody synthesis
3) Adjuvanticity - enhancement
   - suppression
4) Immunogenicity
5) Regulation of mucosal immunity
6) Macrophage activation

For the purposes of this monograph, the most important of these properties are mitogenicity, polyclonal antibody synthesis, adjuvanticity and macrophage activation.

Endotoxins are ubiquitous and no host can fail to be exposed to them (unless it has been maintained in GF conditions). In particular, the gastro-intestinal tract is a rich source of gram negative bacteria continously synthesising endotoxins. Evidence has been presented that gut-derived LPS exerts a negative-regulatory effect on the immune system under normal conditions. It is therefore not a great intellectual leap to accept that, under certain circumstances, aberrant responses to gut-derived endotoxin may be seen.

If LPS gains access to the systemic circulation, the whole of the immune system is potentially exposed to it. Such an exposure may well produce detectable immunological changes. It is well documented that systemic endotoxaemia accompanies a variety of liver diseases. Unrelated studies document immunological changes which could well result from it. These changes include high titres of antibodies to gram negative O antigens and enterobacterial common antigen (E.C.A.), circulating

auto-antibodies, polyclonal elevations in immunoglobulin levels and circulating immune complexes. A causal relationship seems not unlikely.

The reticulo-endothelial system appears to be of crucial importance in the prevention of gut-dervied LPS from spilling over into the systemic circulation and also in removing any LPS that is there. The liver is the most important organ of clearance and detoxification, more specifically, the Kupffer cells which line the hepatic sinusoids. This aspect will be considered in detail in Chapter VII.

Immunological changes which resemble those of chronic liver disease can be observed in the setting of chronic bronchial suppuration (bronchiectasis) and to a lesser extent in association with advanced age and even less following surgical intervention in otherwise normal people. These three clinical situations form the context for the results presented in Chapters IV-VI. Evidence will be presented that the immunological changes described above are seen in each of these situations, and that such chages are associated with detectable lymphocyte activation and possibly with endotoxaemia. Chapter VII is devoted to studies on age-related changes in endotoxin handling in the rat. Chapter II will be devoted to a description of the Limulus test as an assay for endotoxins and Chapter III will summarise the experimental techniques used in a number of the studies cited later.

CHAPTER II

DETECTION AND ASSAY OF ENDOTOXINS USING THE LIMULUS AMOEBOCYTE LYSATE (LAL) TEST

2.1 INTRODUCTION

Two basic approaches may be applied to the detection of any substance of biological interest:

(i) Detection of the substance directly;
(ii) Detection of biological effects.

The first approach is readily applicable to the detection of substances whose structure is relatively simple. When complex structures are involved, chemical or immunological detection of part of the substance may not relate to that activity of the substance which is of interest. Nonetheless, a chemical assay has been described based on the ability of endotoxins to bind and induce a stoichiometric change in the dye Carbocyanine green (Janda & Work, 1971). It is now clear that the binding is specific for toxic sites on the endotoxin molecule and that binding leads to an abolition of toxicity (Ogawa & Kanoh, 1984).

Assays founded on biological effects are often criticised on the grounds of specificity. However, the ability of endotoxins to induce fever has been used by the pharmaceutical industry for a long time (Rabbit Pyrogen Test) and has proved its worth. Quantitative endotoxin bioassays such as the ability to kill appropriately sensitised rodents (lethality assays) are only useful as research tools since several animals are required to assay a single specimen and it must be ensured that a large enough volume of specimen is available to inject into these animals. The only realistic alternative to the Rabbit Pyrogen Test available at present is the Limulus Amoebocyte Lysate (LAL) test. It must however be realised at the outset that correlations between this test and other functional activities of endotoxins are not well established.

2.2 ORIGIN OF THE LAL TEST

This test is based on an interaction between endotoxins and the coagulation proteins of the horseshoe crab. Only four species of this animal can be found today and two of these are widely used in the manufacture of suitable reagents. *Limulus polyphemus* is found along the eastern seaboard of the American continent and *Tachypleus tridentus* along the eastern coasts of Asia, especially Japan. These animals are extremely ancient in evolutionary terms. An easily recognisable ancestor (*Aglaspida*) swam in Cambrian seas while *Mesolimulus* of the Jurassic

period is hardly distinguishable from the horseshoe crabs of today.

The first description of blood coagulation in Limulus was published by Howell in 1885 in the Johns Hopkins University Circular. In 1887, Leo Loeb demonstrated a critical role for the single blood cell type (haemocytes, otherwise known as amoebocytes) in the coagulation process. It is interesting to note that in these animals, the clotting proteins are stored in cytoplasmic granules while the respiratory pigment (haemocyanin) circulates free in the "plasma". It was Frederick Bang who first discovered that endotoxin was able to induce coagulation of Limulus blood. In Bang's own words (1978):

> "In recounting the story of Limulus, I wish to correct any misconception that I had any remarkable inspiration ..... During the course of some naive experiments I was performing at Woods Hole about thirty years ago, I turned from oysters on the half shell, to injecting Limulus with different bacteria. I attempted to determine whether one could observe phagocytosis of bacteria after they were injected..... It soon became obvious that another type of bacterium was present in the animals, and that this Vibrio spp. was capable of inducing disseminated intravascular coagulation".

Bang then went on to compare this with the Shwartzman reaction in mammals (described to him by Jules Freund) which is induced by injecting endotoxin into suitably sensitised animals.

> "To further evaluate this phenomenon, I added some bacteria to a sample of Limulus blood (which contained amoebocytes) to determine if the bacteria could be killed. Within an hour or so, a coagulum occurred which became whitish in colour .... and remained solid for the remainder of the summer".

Together with Levin, he described a method for preparing an aqueous extract of amoebocytes by hypotonic lysis (hence, Limulus Amoebocyte Lysate). This technique was refined by others and a solvent extraction process added (which enhances LAL sensitivity). Lyophilised preparations using this method are what LAL manufacturers produce on a commercial scale.

Research into the mechanism of the coagulation process procedes apace. In the late 1960's it was known that (under appropriate conditions) the rate of gel formation depended upon the concentration of endotoxin present. This reaction appears to take place in two stages; first, activation of a pro-enzyme(s) by

endotoxin (Young et al., 1972) followed by cleavage of internal peptide bonds of a coagulogen, thus converting it into an insoluble form (Tai et al., 1977). Further research has revealed that the coagulation system is somewhat more complex (Fig.2.1) and can perhaps be compared with human blood coagulation (Morita et al., 1984).

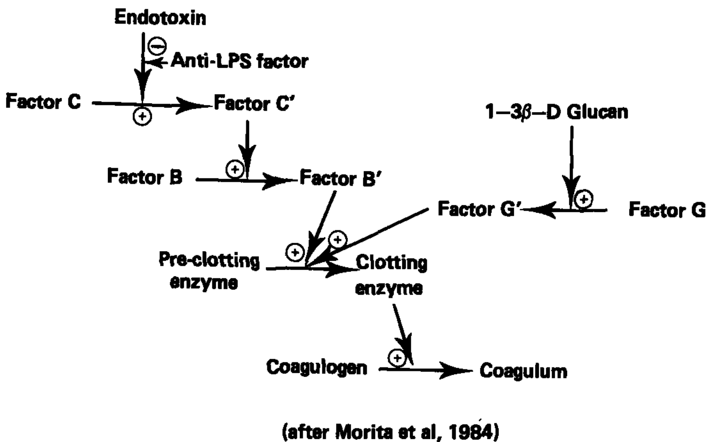

Fig.2.1 Coagulation system of the Horseshoe Crab.
+ = stimulatory
- = inhibitory

2.3 REFINEMENT OF THE LAL TEST

As described so far, the test is solely qualitative but results obtained by the application of this procedure were in substantial agreement with the Rabbit Pyrogen Test (Levin, 1982). It soon became clear that changes in turbidity in the reaction mixture depended both on time and endotoxin concentration. Measurement of turbidy gave poor standard curves on "end-point" analysis but kinetic analysis gives very reproducible standard curves (Sloyer et al., 1982). Such analysis has the added advantage that the original colour of the reaction mixture pales into insignificance.

An alternative method of assessing the LAL-endotoxin interaction became possible with the observation that activated LAL could cleave 4-nitroaniline from

some synthetic chromogenic substrates (Nakamura et al., 1977) and that the intensity of the colour so generated is proportional to the amount of endotoxin present (Iwanaga et al., 1978). This means of detection is amenable to both kinetic and end-point determinations.

One new strategy has recently been described employing rocket immunoelectrophoresis for the detection of the products of LAL activation (Baek, 1983). Insufficient information has, as yet, accrued to evaluate this test in practical terms.

2.4 USES AND LIMITATIONS OF LAL TESTS

The practical use of the LAL test was first taken up by the pharmaceutical industry as a possible alternative to the Rabbit Pyrogen Test. Possible applications are outlined in Table 2.1. United States Federal Authorities now permit the use of a LAL test (under strictly defined conditions) as an alternative to the Rabbit Pyrogen Test. The Food and Drug Administration (FDA) guidelines are summarised by Munson (1982). The protocol described in the U.S. *Pharmacopeia* now requires results to be given in terms of units of potency (Section 2.5) based on the United States Reference Standard Endotoxin (RSE).

TABLE 2.1. APPLICATIONS OF THE LAL TEST (after Levin, 1982)

Applications
Pharmaceutical preparations
Radioisotopes
Water supplies
Intravenous fluids
Biological reagents
Body fluids and their products

A number of studies have criticised the validity of the LAL test in relation to the testing of blood products, both on the grounds of sensitivity and specificity. Problems regarding sensitivity relate to the failure of endotoxin recovery from serum and plasma samples "spiked" with endotoxin. It was later shown that both serum and plasma contain substances which may interfere with the LAL reaction, and a number of these have been characterised (Table 2.2). Levin (1970a) employed a chloroform extraction procedure to overcome this problem and a number of other methods have been described since (e.g. perchloric acid treatment, dilution, dilution plus heating). Further doubts were raised in studies evaluating the LAL test as an adjunct to the diagnosis of gram negative septicaemia.

TABLE 2.2 INHIBITORS AND ACTIVATORS OF THE LAL TEST (after Levin, 1982)

INHIBITORS	α_1 - globulins
	α_1 - lipoprotein esterases
	anti - endotoxin antibodies
	α_1 - antitrypsin
	α_2 - macroglobulin
	antithrombin III
	(heparin)
ACTIVATORS	thrombin
	plasmin
	(heparin)

One study by Stumacher and co-workers (1973) reported the results of 694 LAL tests in 344 episodes of suspected gram negative septicaemia. Positive tests were found in 43% of cases where gram negative septicaemia was subsequently proven; in 26% of those with localised gram negative infections; and in 36% of those with serious gram positive infections. One can criticise this study (and others like it) since they appear to have been performed on the basis of two preconceptions, which then led the authors to doubt the value of the LAL test:

1) That the presence of gram negative bacteria in blood could be the only explanation for the presence of endotoxins;
2) That endotoxins should always be detected by the LAL test when bacteria were present.

In answer to the first preconception, it seems unreasonable to assume that the presence of bacteria in blood can be the only reason for the finding of endotoxin in that body fluid. It has been calculated that some 400,000 ng of endotoxins are generated daily in the human colon and endotoxins are ingested in food and water. Evidence has already been given (Chapter 1) that some of this appears as a usual constituent of portal venous blood and may (in certain situations) spill over into the systemic circulation. Furthermore, endotoxin must be released in areas of localised gram negative infection (by bacterial growth and lysis) and the vascular supply of most such areas is entirely systemic. Endotoxin present in venous blood or lymph would then be discharged directly into the systemic circulation.

In answer to the second point, one can calculate that more than 100 organisms/ml of blood must be present to produce a positive LAL test. Our best estimates suggest that an average gram negative bacterium contains some 30-40 fg of endotoxin. Even 100 such organisms per millilitre would elude the limit of resolution of all but the most sensitive LAL tests (about 5 pg/ml).

Doubts about specificity relate to claims that a number of substances can activate LAL. One of these is 1-3β-D glucan (a constituent of gram positive cell walls, see Fig.2.1). It is often (conveniently!) forgotten that, on a weight for weight basis, between 1000 and 400,000 times more 1-3-βD-glucan is needed than endotoxin. This means that contamination with endotoxin at a level of 0.1% - 0.00025% could explain the positive tests.

Claims have also been made that some biological reagents can also activate LAL. Rickles (1981) assessed ten batches of concanavalin A by both the LAL test and the Rabbit Pyrogen Test. Nine batches failed both tests and one batch passed both. The only reasonable conclusion is not that concanavlin A activates LAL, but that it is frequently contaminated with endotoxin. Likewise, we have found at the Institute for Experimental Gerontology that all our batches of neonatal calf serum were heavily contaminated with endotoxin (tens of nanograms/ml) and that very stringent conditions must be employed to ensure that such protein preparations remain endotoxin free.

In conclusion, it may reasonably be assumed that the LAL test is both sensitive and specific provided that the conditions under which the test is used are well defined and understood. Because of the ubiquity of endotoxins, extreme effort must be made to ensure that they are not inadvertently introduced into any step of an experimental procedure.

2.5 STANDARDISATION OF THE LAL TEST

Endotoxin preparations from different bacterial species and strains vary widely in their potency as do the products of different extraction procedures (Outschoorn, 1982). Standardisation is therefore imperative! The FDA set about doing just that. Their requirements for an endotoxin standard were that it must be of intermediate potency (so that a wide range of preparations could be tested against it); that it should contain no di-deoxyhexoses (in case later chemical analysis with measurement of keto-deoxy octulosinic acid was required); and that the standard should remain stable for prolonged periods without the need for too exacting storage conditions. They eventually selected E.coli O113:H10:K-negative bacteria for use as a reference standard endotoxin (RSE). The RSE produced was designated EC (from E.coli). The Endotoxin Unit of potency (EU) was introduced during the reign of the second lot of RSE, designated EC2; 1 EU being equivalent in potency to 0.2 ng of EC2. The currently available RSE (EC5) is labeled solely in terms of units of potency. The widespread acceptance of this unit of potency (EU) would facilitate comparison of results between different laboratories.

For the purpose of this monograph, the studies in man employed a standard with a potency of 0.0115 EU/pg while the potency of the standard used for the animal studies was 0.0125 EU/pg.

Notwithstanding the variation in potency between different endotoxin prepara-

tions, preparations of LAL also vary in their sensitivity. Furthermore, endotoxins which appear equipotent with one LAL preparation are not necessarily equipotent with another. Using a LAL gel-clot test, variation by a factor of 2 is acceptable while with chromogenic assays, such variation must not exceed 10%. The calculated sensitivity (by gel test) of the LAL used in these studies (results not shown) is 0.075 EU/ml.

2.6 THE LAL GEL-CLOT TEST

2.6.1 Introduction

Our first publication employing this test employed chloroform extraction (after the method of Nandan et al., 1977) to remove inhibition and samples were processed on the day they were obtained (Horan et al., 1984). In addition, duplicate samples were frozen at -70°C and tested later following treatment with perchloric acid. The validity of storage in this way will be established in Section 2.7.4. The results of the tests following perchloric acid treatment did not differ from those obtained after chloroform extraction and therefore, only the former method is described here.

2.6.2 Sample Preparation

Five millilitres of venous blood were collected aseptically into disposable plastic syringes which had been rendered sterile and pyrogen-free by gamma irradiation. The blood was discharged into radiation sterilised Falcon tubes containing 50 units of heparin. The heparin had been previously tested over a range of dilutions and established to be negative in the LAL test. Samples were kept on ice until centrifuged under 200 g at 4°C for ten minutes. Four hundred microlitres of 1.9% perchloric acid were added to 200 µl of plasma and the mixture incubated at 37°C for 20 minutes. After centrifugation under 250 g for 15 minutes, 200 µl of the supernatant was added to 200 µl of 0.2 M sodium hydroxide and the resulting solution (plasma diluted 1:4) was used as the sample for the LAL test.

2.6.3 Performing the Gel-Clot Test

Two hundred microlitres of treated plasma was added to vials containing LAL (M.A. Bioproducts, Walkersville, MD, USA) and after mixing, vials were maintained at 37°C for four hours and checked at hourly intervals for gel formation. Samples in which no gel had formed by four hours were then maintained at 25°C for a further 18 hours and checked for gel formation at the end of this time. Each batch of patient samples was run with at least two samples obtained from normal (medical student!) "volunteers".

2.7 THE "AUGMENTED" CHROMOGENIC LAL TEST

2.7.1 Introduction

At the time we wished to experiment with a chromogenic LAL test, a reagent set had recently come onto the market (M.A. Bioproducts, Walkersville, MD, USA). This set comprises:

- seven vials of lyophilised LAL (each to be reconstituted with 1.4 ml of sterile, pyrogen-free distilled water);
- substrate buffer for use with the chromogenic substrate;
- endotoxin standard (E.coli 0111:B4), 23 EU;
- sterile, pyrogen-free water.

The chromogenic substrate, S 2423, was obtained from Kabi Vitrum (Stockholm, Sweden) and dissolved in water from the reagent set to a final concentration of 2.2 mM. Prior to use in the assay, an equal volume of this solution was added to the substrate buffer and kept at 37°C, at which temperature it is stable for several hours. The test described by the manufacturers proved too expensive for us to use but it could be adapted to a "micro-method" by scaling down the volumes of reagents used. Unfortunately, this would have involved our investing in expensive new equipment. However, a method had recently been described for amplifying the colour intensity from the chromogenic substrates S 2227 (Kabi Vitrum) and chromozym (Pentapharm AG, Basel, Switzerland). This method (described by Kwaan et al., 1978) was therefore adapted for use with the LAL test which, in effect, allowed us to use a "micro-assay" without the need for micro-equipment. This new test was designated "the augmented chromogenic LAL test".

The aromatic amine 4-nitroaniline (PNA), released from chromogenic substrates is coupled to an aldehyde (4-dimethylaminocinnamaldehyde, DACA) to form a Schiff's base which has a maximal absorbance at 578 nm. Using the data provided in Kwaan's paper it proved relatively simple to adapt the technique to the chromogenic LAL test (data not shown). The nature of this reaction is illustrated in Figure 2.2.

2.7.2 Augmented Chromogenic LAL Test in Water

All glassware was rendered sterile and pyrogen-free by overnight dry heating at 180°C. Individually wrapped, radiation sterilised, disposable plastic pipette tips were used throughout (Gilson or Eppendorf). All procedures were performed in a Class II microbiological safety cabinet. Incubations were performed in a water bath held at 37°C (unless otherwise stated).

Standard curves were constructed based on endotoxin standards of 0, 10, 20, 50, 80 and 100 pg/ml (0 - 1.15 EU/ml). Standards were vigorously vortexed for three minutes and then 50 µl pipetted into each of up to 18 glass tubes. After all standard samples were ready, starting at time 0, tubes were placed in a water

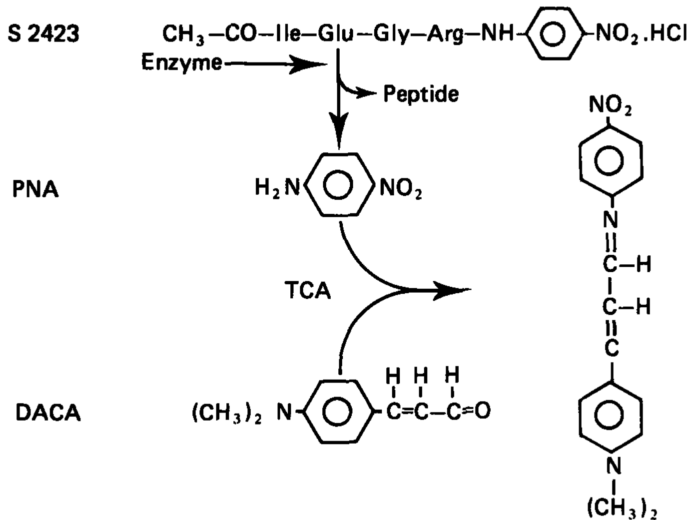

Fig.2.2 Amplification of the colour yield from the chromogenic substrate S 2423 by forming a Schiff's base under acid conditions (Trichloroacetic acid, TCA) with dimethylaminocinnamaldehyde (DACA).

bath at ten second intervals. At time four minutes, an equal volume of freshly reconstituted LAL was added to each tube, also at 10 second intervals. At time 14 minutes, 100 l of chromogenic substrate in buffer was added (also at ten second intervals) and the incubation allowed to proceed until time 17 minutes when the reaction was stopped by the addition of 400 µl of 40% trichloroacetic acid (TCA), also at ten second intervals. Two hundred and fifty microlitres of an alcoholic solution of DACA (8 mg/ml) was added to each tube which, after mixing, was allowed to stand at room temperature for at least 15 minutes, though the colour change remains stable for several hours. The total volume generated during this test is 850 µl, more than enough for use with semi-micro cuvettes. Absorbance was read at 578 nm and standard curves plotted. The coefficient of correlation was determined with a Hewlett-Packard HP-41 CV programmable calculator. With practice, the "r" value is always in excess of 0.95, and usually 0.98 or better.

This procedure was repeated for 20 consecutive standard curves and the coefficient of variation for each point was calculated. The results are illustrated in

Figure 2.3. The within assay coefficient of variation was determined for each point on the above standard curve and ranged between 3-8%.

Comparable results (not shown) were obtained over the range 0-10 pg/ml by extending the initial incubation with LAL from 10 minutes to 25 minutes, and the incubation with substrate from 3 minutes to 6 minutes.

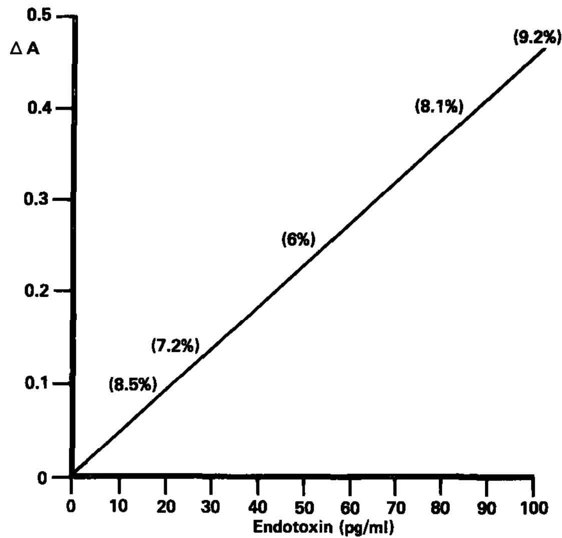

Fig.2.3 A synthesis of twenty consecutive standard curves derived by linear regression analysis showing the "between assay" coefficient of variation for each point on the curve.

2.7.3 The Augmented Chromogenic LAL Test in Plasma

Dilution and heating were chosen to remove plasma interference, largely by virtue of simplicity. Platelet rich plasma was prepared from the heparinised blood of ten normal subjects and "spiked" over a range of plasma dilutions (in water) over the range 0-25% plasma with 50 pg/ml of the standard endotoxin. After vigorous vortexing for five minutes, each sample was split into two aliquots and one sample from each pair was heated for ten minutes at 75°C. The test was then performed

as described in section 2.7.2 and the results expressed as a percentage of the "spiked" sample containing no plasma. The results are given in Fig. 2.4. It was concluded that a 1 in 10 dilution of plasma (plasma concentration 10% v/v), as recommended by the manufacturer, was an adequate precaution against plasma inhibition.

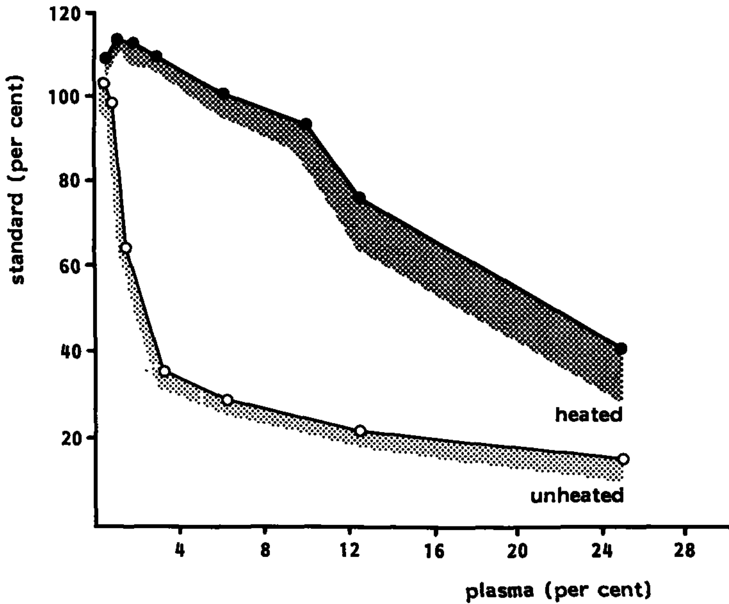

Fig.2.4 Effect of plasma dilution and heating at 75°C for 10 minutes on overcoming plasma inhibition in the augmented chromogenic LAL test.

2.7.4 Stability of Endotoxin in Plasma

This question was raised since it was desirable to store samples for later assay in batches. To assess this possibility, a 10% aqueous solution of plasma was spiked with endotoxin to a concentration of 50 pg/ml. After heating at 75°C for ten minutes, cooling and vigorous vortexing, 50 l of the preparations were dispensed into tubes and then stored at either -20°C or -70°C. Two samples were tested at approximately fortnightly intervals for a total of three months. At the end of this period, the coefficient of variation was calculated and proved to be well within the limits set for the assay (7% vs 10%). It was therefore concluded that heat-treated, diluted plasma samples could be kept for at least three months at either -20°C or -70°C.

2.7.5 Stability of LAL

Following a series of assays, it was a common occurrence that a significant amount of the expensive LAL reagent remained. It was therefore decided to determine whether it retained its activity on storage.

One vial of LAL was reconstituted and stored in 50µl aliquots at -70°C. Two vials were tested at monthly intervals against a 50 pg/ml endotoxin standard. After nine months, the results were analysed and a coefficient of variation of only 10% obtained. It appears, therefore, that there is no significant loss in activity when LAL is stored under these conditions for at least nine months.

2.7.6 Comments on the Augmented Chromogenic LAL Test

It can be concluded that the augmented chromogenic LAL test, as described above, performs as well as other chromogenic LAL tests. When applied to human plasma samples, the standard curve is plotted over the range 0-10 pg/ml and both the LAL incubation step and the chromogenic substrate reagent step extended to 25 minutes and 6 minutes, respectively. A combination of 1:10 dilution and heating at 75°C for ten minutes appears an adequate precaution against plasma interference. One disadvantage is that plasma samples must be centrifuged prior to the measurement of absorbance, since the TCA precipitates protein. The LAL test is more sensitive than any other endotoxin assay. It is readily amenable to testing multiple samples, thus avoiding unnecessary use of laboratory animals. Since there is no more sensitive yardstick against which the LAL test can be assessed, the specificity of the test cannot be stated in absolute terms.

2.8 THE "NON-AUGMENTED" CHROMOGENIC LAL TEST

This test was employed for endotoxin estimation in the experiments utilising laboratory animals. The reagent set (obtained from Kabi Vitrum, Stockholm, Sweden) was identical to the one described above except that it already contained the chromogenic substrate, S2423. Tests were done in an electric heating block housed in a Class II microbiological safety cabinet. The volumes used in the test were as follows:

Diluted plasma	30 µl
LAL	30 µl
Substrate + buffer	60 µl
50% acetic acid	60 µl

The test sequence was performed as is described in section 2.7.2 except that the reaction was stopped with 50% acetic acid. Samples were then transferred to plastic microtitre plates and absorbance read in a microplate reader. Standard curves were constructed as described in previous sections. This test always produ-

ced standard curves with a correlation coefficient of 0.98 or better.

Incubation times depended upon the sensitivity required for the experiment in question. Plasma inhibition curves were constructed, as described in section 2.7.3. The results obtained from eight rhesus monkey plasma samples are given in Fig. 2.5. Rat plasma gave results very similar to those obtained from humans.

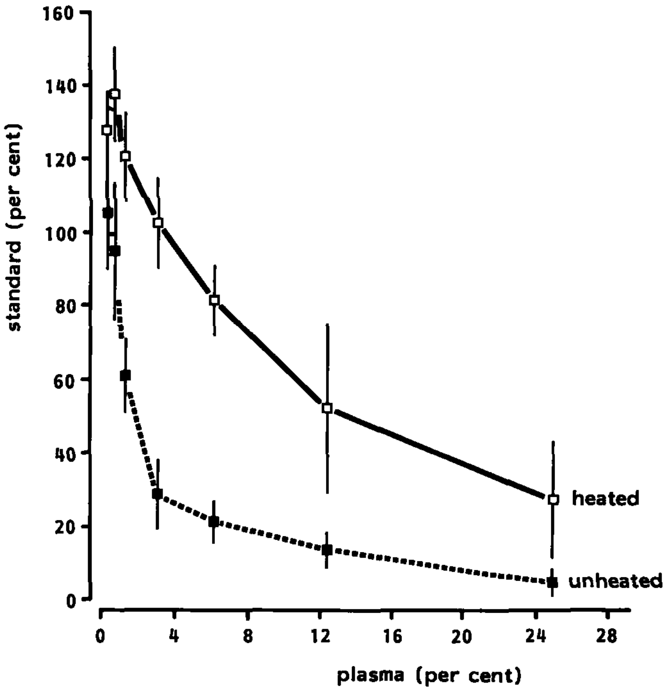

Fig. 2.5 Effect of plasma dilution and heating at 75°C for ten minutes on overcoming plasma inhibition in the chromogenic LAL test for eight rhesus monkey plasma samples.

CHAPTER III

METHODS

3.1 INTRODUCTION

A number of the studies to be described in this monograph depend upon the use of certain common methods. For the sake of brevity, these methods will be described but once (vide infra). Techniques which are peculiar to one particular study will be described in the appropriate place.
All blood samples for immunological investigations were collected between 08.00 and 12.00 hrs to avoid the possible complicating factor of circadian variation.

3.2 SEPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS AND DETECTION OF SURFACE ANTIGENS

3.2.1 Separation of Mononuclear Cells

All disposable plastic utensils used had been rendered sterile and pyrogen-free by gamma irradiation. Peripheral venous blood was drawn into disposable syringes and the contents discharged into plastic universal containers containing 100 units of endotoxin-free heparin. Specimens were maintained at 4°C until further processed.

After 1:1 dilution in phosphate buffered saline (PBS), the blood was layered on Lymphocyte Separation Medium (Flow Laboratories) and centrifuged (after the method of Bøyum, 1968). Interface cells were harvested and washed thrice in PBS before being suspended in medium RPMI supplemented with glutamine (± serum, as appropriate).

3.2.2 Detection of Membrane Antigens

Detection of membrane antigens was performed during experiments which were designed to produce a method for detecting membrane antigens on autoradiographically positive cells. The first step was to perform a classical immunofluorescence technique and compare it with an immuno-cytochemical slide technique. Since no success was achieved with the immuno-cytochemical method for identifying B lymphocytes, further discussions on B lymphocytes will be avoided.

3.2.2.1 Immunofluorescence

Indirect immunofluorescence was performed by the Department of Immunology, Hope Hospital, Salford. OKT3, OKT4 and OKT8 monoclonal antibodies (Ortho

Diagnostic Systems Ltd.) and fluorescein-labeled goat anti-mouse immunoglobulin (*Nordic Immunological Laboratories, Tilburg, The Netherlands*) were used. The antibody OKT3 is a pan-T cell marker, OKT4 recognises T helper cells and OKT8 recognises T suppressor/cytotoxic cells.
Mononuclear cell populations were prepared as described under 3.2.1. Tests were performed at 4°C using 10^6 mononuclear cells with greater than 95% viability (as determined by Trypan blue exclusion). Cells were counted using a Leitz dialux microscope with a x50 objective. Two hundred cells per test were counted and only cells of lymphocyte morphology were included.

3.2.2.2 Sheep Red Blood Cell (SRBC) rosette formation

T cells were also detected by utilising their ability to form rosettes with Sheep Red Blood Cells (SRBC's). Mononuclear cells were incubated overnight at 4°C with neuraminidase-treated (BDH Chemicals Ltd.; 12.5 u/ml for 30 minutes) SRBC's. All samples were tested in triplicate.

3.2.2.3 Immuno-cytochemical slide technique

The monoclonal antibodies OKT3, OKT4 and OKT8 (Ortho Diagnostic Systems Ltd.) and affinity-purified, peroxidase conjugated sheep anti-mouse immunoglobulin (Amersham International) were used. Five microlitres of reconstituted monoclonal antibody were added to siliconised glass tubes containing 8×10^5 mononuclear cells. Two control tubes were also used. After 40 minutes incubation at 4°C in medium RPMI, cells were washed thrice in PBS. They were then treated with 0.3% hydrogen peroxide in medium RPMI for five minutes (to deplete endogenous peroxidase activity) and then washed twice more. The cells were suspended in 200 L of medium RPMI and incubated at 4°C with the peroxidase-conjugated anti-mouse immunoglobulin (100 µl of a 1:1000 PBS dilution) for 15 minutes. Conjugate was omitted from one of the control tubes, At the end of this period, cells were washed thrice more and cytocentrifuged onto glass microscope slides and allowed to dry.

The substrate 3,3'-diaminobenzidine (DAB) was chosen as electron donor. Fifty milligrams were dissolved in 100 ml of PBS and 100 µl of 30% hydrogen peroxide added immediately before use. Slides were immersed in this solution for two minutes, then gently washed in PBS. Counterstaining with 1% methyl green was performed before taking the slides through a graded alcohol series followed by xylene. The preparations were mounted in DPX under glass coverslips and examined under the microscope. Positively-reacting cells display brown linear deposition of substrate along the cell margins (Fig.3.1a). This is easily distinguished from the diffuse, granular deposition in cells in which endogenous peroxidase activity had not been completely blocked. For the purposes of the study, a total of 200 cells were counted and the proportion of positive cells expressed as a percentage of this total.

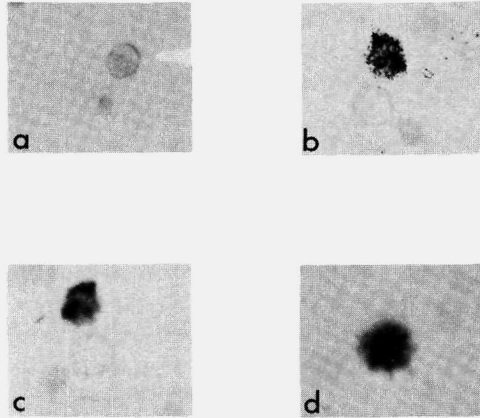

Fig.3.1

a) Two cells can be seen displaying linear deposition of substrate, indicating a positive reaction with the monoclonal antibody OKT3 (1% methyl green counter-stain).

b) Combined autoradiography and immunocytochemical preparation demonstrating an autoradiographically positive cell and below which can be seen an auto-radiographically negative cell which has been recognised by the monoclonal antibody OKT3. An erythrocyte contaminant can be seen below and to the right of this cell.

c) From the same preparation as 3.1b except that the microscope is focussed below the plane of the silver grains.

d) An autoradiographically positive cell which is also recognised by the monoclonal antibody OKT3. Note the dark rim which is seen most clearly around the upper border of the cell.

Results from this immunocytochemical method were compared with those from immunofluorescence and the two methods compared by linear regression analysis utilising the statistics functions of a Hewlett-Packard 41-CV programmable calculator. A correlation coefficient of 0.89 was obtained and it was therefore concluded that the two methods gave comparable results.

3.3 SPONTANEOUS INCORPORATION OF TRITIATED THYMIDINE

Two methods were compared to assess this parameter, namely autoradiography and liquid scintillation counting.

3.3.1 Liquid Scintillation Counting

Mononuclear cells were isolated as described under 3.2.1. and suspended in medium RPMI at a concentration of 2.5×10^6/ml supplemented with glutamine and 10% heat-inactivated pooled human serum (PHS) . One hundred microlitre aliquots were dispensed into the wells of round-bottomed microtitre plates (Flow Laboratories) and 1 µCi of tritiated thymidine (specific activity 6.7 Ci/mmol, Amersham International) in 150 µl of RPMI, was added. Plates were incubated at 37°C in humidified air supplemented with carbon dioxide (5%) for a period of two hours. At the end of this period, cells were harvested onto glass fibre filters using an automated millipore cell harvester. Thymidine incorporation was assessed in a liquid scintillation counter (LKB 81000) and the results expressed in counts per minute (after the subtraction of background counts).

3.3.2 Autoradiography

For each patient sample, four wells were set up under the conditions described in 3.3.1. After the period of incubation, the cells were resuspended and transferred to small, siliconised glass tubes and washed three times in PBS. After the final wash, they were suspended in 50 µl of PBS and cytocentrifuged onto glass microscope slides, stained with 1% methyl green, dried and fixed in alcohol. Slides were stored in trays at 4°C until enough were prepared for autoradiography.

Plates of Kodak AR 10m stripping film were subjected to saturated water vapour in a darkroom (illuminated only by a red safelight) for at least two hours. The film was then divided into sixteen pieces using a sharp scalpel blade and each piece floated onto the surface of dust-free distilled water maintained at a temperature of 23-25°C and left to swell for three minutes (emulsion side downwards). Each piece of film was then picked up onto the microscope slides bearing mononuclear cells, so that the cytocentrifuged cells were in contact with the emulsion. After drying in air, slides were exposed at 4°C in light-proof boxes. An exposure time of 7-8 days was found to be optimal.

Exposed slides were developed in Kodak D19b developer for four minutes, then washed in water for 15 seconds. Fixation was achieved by immersion in a 15% solution of anhydrous sodium thiosulphate. After washing in gently running water for 30 minutes, the slides were dried in air and mounted under glass coverslips in DPX.

Slides were examined under the microscope and 3000 cells (excluding any red cell contaminants) were counted. Cells with more than six grains were scored as

positive (though in the majority of cases the grains were confluent, see Fig. 3.1.b). The proportion of autoradiographically positive cells was expressed as a percentage.

3.3.3 Comparison between the Two Techniques

Fifteen autoradiographs were examined "blind" over a range of scintillation count values. The results were submitted to linear regression analysis and are shown in Figure 3.2. (r = 0.91).

In a single individual (the author!), the above tests were performed at approximately weekly intervals for about 5 months. As can be seen from Figure 3.3, there was little variation in the scintillation count values during the period of observation in that values were maintained within the 200-400 cpm range, except during a Mycoplasma pneumonia respiratory tract infection (proven serologically).

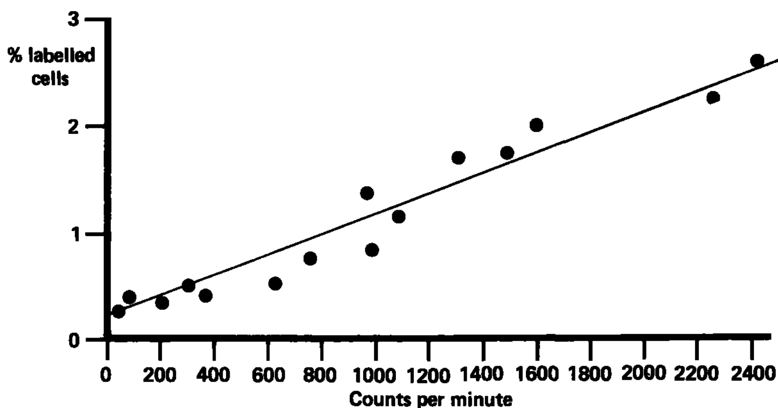

Fig.3.2 Comparison between autoradiography and liquid scintillation counting to identify "spontaneously active" peripheral blood mononuclear cells. This figure may be used to convert the scintillation count values from later chapters to give an approximation to the percentage of DNA synthesising cells.

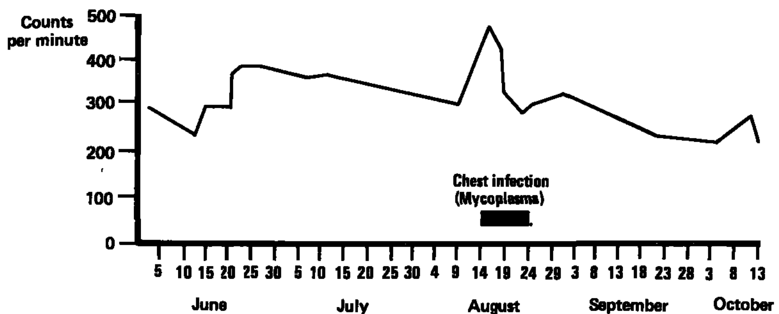

Fig.3.3 Scintillation count values representing "spontaneously active". mononuclear cells in a single individual over a five-month period.

3.4 IDENTIFICATION OF "SPONTANEOUSLY" ACTIVE (UNSTIMULATED, DNA SYNTHESISING) MONONUCLEAR CELLS

This was done by combining the autoradiography and immunocytochemical techniques described above. Mononuclear cell suspensions (2.5×10^6/ml) were prepared and subjected to a two hour incubation with tritiated thymidine under the conditions specified above. At the end of this period the cells were re-suspended, transferred to siliconised glass tubes and washed thrice before being subjected to the immuno-cytochemical methods for surface antigen detection, precisely as described above. Once the slides had been taken up to absolute alcohol, they were stored in trays at 4°C before processing for autoradiography. No counterstain was used.

Preparations were examined under a light microscope and the presence or absence of the surface antigen in question was determined for 100 autoradio-

graphically positive cells (Fig. 3.1.d) in an area of the preparation where good cell separation was achieved. The proportion of "spontaneously active" cells positive for the relevant surface marker was expressed as a percentage of the total number of autoradiographically positive cells. Most normal individuals had so few autoradiographically positive cells that it was impossible to obtain reliable values for surface markers on "spontaneously active" cells in these individuals.

3.5 OTHER IMMUNOLOGICAL TECHNIQUES*

All measurements were made on sera. C-reactive protein and immunoglobulin levels were measured by rate nephelometry (Beckman Immunochemistry Analyser II). The normal ranges for the relevant laboratories are as follows:

C-reactive protein	less than 3 mg/l
IgG	8.0 - 18 g/l
IgA	0.9 - 4.5 g/l
IgM	0.6 - 2.8 g/l

Antibodies to cell nuclei, smooth muscle, mitochondria, reticulin, and gastric parietal cells were detected by indirect immunofluorescence using six cryostat sections of rat liver, kidney and stomach. Sera were screened at a dilution of 1:10 with a fluorescein conjugated polyvalent rabbit antiserum to human immunoglobulins (Wellcome Reagents Ltd.) and positive sera titred using conjugated antisera specific for IgG or IgM (Wellcome Reagents Ltd.). Rheumatoid factor was detected by latex agglutination (Brocades Ltd.) and by sheep cell agglutination (RAHA, Fujizoki Co., Ltd.).

Fibronectin was measured by radial immunodiffusion** using a monospecific antiserum to fibronectin in agarose (Serotec). These measurements were performed using citrated plasma. The final values were calculated after correction for the dilution factor.

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* These tests were performed by the Department of Immunology, Hope Hospital, Salford and the Regional Immunology Laboratory, St. Mary's Hospital, Manchester.

** Performed by Mrs. Diane Johnston, Regional Immunology Laboratory, St. Mary's Hospital, Manchester.

CHAPTER IV

IMMUNOLOGICAL ASPECTS OF CHRONIC BRONCHIAL SUPPURATION

4.1 INTRODUCTION

4.1.1 Structure and Function of the Respiratory Tract

The larynx is conventionally considered to mark the frontier between the upper and lower respiratory tracts. It opens into the trachea which bifurcates shortly after entering the thorax, giving rise to two main bronchi which enter the left and right lung respectively. In humans, deep fissures divide the lungs into upper and lower lobes. In addition, a transverse fissure separates off a middle lobe on the right side only. The air passages branch within the lungs some 8-24 times depending on the relative distance between the hilum and the pleural surface. Cartilage-containing air passages distal to the trachea are termed bronchi, while the airways distal to the last cartilage plate are designated bronchioles. In the last divisions, the ciliated lining epithelium is interrupted by thin-walled sacs called alveoli. This is the first site of gas exchange and the place where the muco-ciliary escalator (see later) begins. These airways are called respiratory bronchioles and the branches immediately proximal to them, the terminal bronchioles. The terminal bronchiole with its subsequent divisions is often referred to as a functional ventilatory unit (Fig. 4.1)

The alveolar surface provides an enormous area accross which gas exchange occurs. This is facilitated by a rich, low pressure, vascular network which ultimately derives from the pulmonary artery. The pulmonary artery carries the entire output of deoxygenated blood from the right ventricle. The lungs also receive a minor vascular contribution of oxygenated blood from the aorta via bronchial arteries.

Although gas exchange is the main "raison d'être" of the lungs, they also fulfill a number of metabolic functions. For example, acetyl choline is completely inactivated by a single passage through the lungs. Furthermore, it is believed that bradykinin, 5-hydroxy tryptamine and some prostaglandins are inactivated exclusively by the lungs. The lungs also synthesise angiotensin converting enzyme (ACE) which converts angiotensin I to the highly active angiotensin II. The lung also manufactures a number of protease inhibitors which may play an important role in the defence of the lung against endogenous or exogenous (e.g. bacterial) proteases. Genetic defects in protease inhibitor production (e.g. α_1-antitrypsin deficiency) lead to severe, progressive basal emphysema.

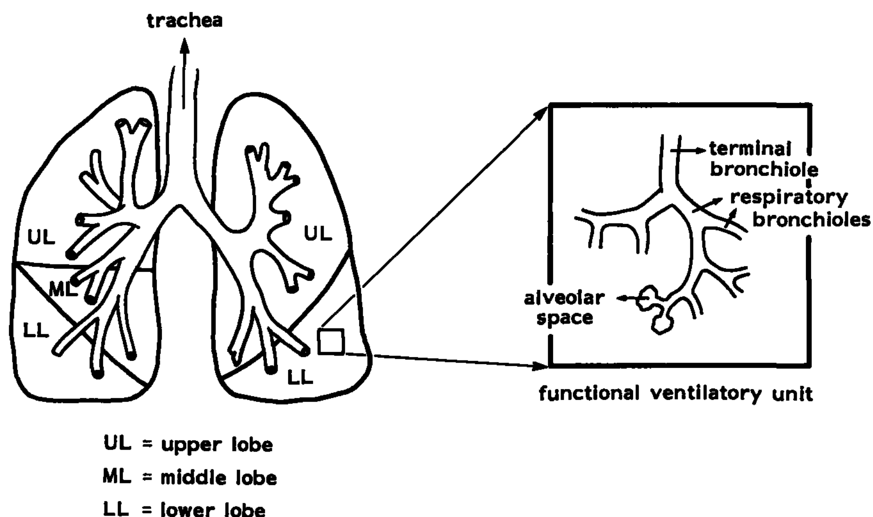

Fig. 4.1 Structure of the human tracheo-bronchial tree.

4.1.2 Non-immune Pulmonary Defences

Particle filtration is a function of the lower respiratory tract. The probability of particle impaction depends on both particle size and particle density. Larger and more dense particles are more likely to impact. These two variables may be combined to produce a term known as the aerodynamic radius. Particles with an aerodynamic radius 30 µm are trapped within the nasopharynx. Particles from 10-30 µm are removed in the upper tracheo-bronchial tree. Particles from 0.3-2 µm enter the alveoli and tend to remain in suspension. Particles in the 2-10 µm range tend to impact at the sites of bifurcation within the bronchial tree and the probability of impaction is proportional to the cosine of the angle between parent and daughter branches. It is interesting to note that lymphoid aggregates are found at these sites of bifurcation.

Impacted particles which do not confront lymphoid tissue are trapped in a layer of mucus which is continuously moved upwards by the action of cilia (muco-ciliary escalator) and, on reaching the mouth, are either swallowed or expectorated. In patients with the immotile cilia syndrome (Kartagener's syndrome), bronchiectasis frequently develops. Furthermore, bacteria may release ciliotoxic substances, thus directly impairing the muco-ciliary escalator (Wilson et al., 1985).

4.1.3 Immune Defences

A variety of immunologically active cell types are associated with the pulmonary parenchyma and the air spaces. Lymphocytes can be found scattered throughout the epithelium, sometimes organised into a lympho-epithelium. Mast cells probably account for up to 1% of the cells in the epithelial lining in the human lung. However, the bulk of the lung mast cells reside below the epithelium and closely resemble the epithelial mast cells.

Lymphoreticular aggregates occur throughout the lungs, especially around bronchi, terminal bronchioles and respiratory bronchioles. Lymphocytes are the predominant cell type in these aggregates, though small numbers of plasma cells and eosinophilic granulocytes are also found. A fine connective tissue network can be demonstrated by reticulin stains but sinusoidal and nodal architecture is absent.

Bronchial-associated lymphoid tissue (BALT) is especially associated with bronchial bifurcations. It is structurally very similar to the gut-associated lymphoid tissue (GALT) and contains all the cell types needed to induce an immune response. Lymph nodes are associated with large bronchi and are found within the peri-bronchial tissues. These nodes eventually drain via lymphatic vessels to the mediastinal lymph nodes.

Within the alveolar spaces, the main cell type is the alveolar macrophage which resides within the alveolar lining film. These cells employ aerobic metabolism (in contrast to peritoneal macrophages), they can present antigens, they can respond to lymphokines and their activity is enhanced by surfactant (which is also chemotactic for them). The ultimate fate of alveolar macrophages remains controversial, though the consensus view is that the majority ascend the muco-ciliary escalator and a minority penetrate the interstitium.

Immune secretory products can be detected in airway secretions. IgA accounts for some 5% of the protein in broncho-alveolar lavage fluid (90% of which is dimeric, secretory IgA). IgG accounts for around 14% while IgM and IgE are found in only trace amounts (Young & Reynolds, 1984). Specific antibody responses can be detected to inhaled antigens and likewise, hypersensitivity phenomena can contribute to pathological states (Willoughby & Willoughby, 1984). This lends support to the concept that BALT functions somewhat independently as a compartment of the immune system in much the same way as does GALT.

4.1.4 Endotoxins and the Lung

Systemically administered endotoxin preparations lead to pulmonary release of thromboxanes and prostaglandins during the first hour of infusion in sheep (Ogletree & Brigham, 1982). This is associated with severe pulmonary hypertension (so-called "phase I"). This is followed by a prolonged period of increased pulmonary vascular permeability with less marked elevations in pulmonary artery pressure (phase II). _In vitro_ studies however, have shown that alveolar macrophages

are both capable of taking up endotoxin and degrading it (Filkins, 1970). Guinea pigs acutely exposed to endotoxin by inhalation (by aerosol) showed a decrease in the activity of a number of alveolar macrophage enzymes within hours and which continued to decline up to about 24-30 hours and thereafter returned to normal (Rylander et al., 1983). Other studies have shown that endotoxin protected the lung against oxygen-induced lung damage (Frank & Roberts, 1979). No studies have been performed on the distribution and uptake of inhaled endotoxins.

Inhaled endotoxin produces migration of neutrophil granulocytes into the airways (Bomski et al., 1971). Similar observations have been made in a variety of animal species experimentally (Helander et al., 1980). Whether this effect is directly induced by endotoxins or mediated by products of alveolar macrophages is not yet resolved. Inhaled endotoxins certainly induce fever and it has been suggested that this is of clinical relevance in "cotton workers lung", "mattress makers lung" and humidifier fever (Helander, 1982). The proposed central mechanism is interleukin 1 release from alveolar macrophages (see Chapter I). No studies have been performed assessing other immunological parameters following endotoxin inhalation.

Acute exposure to inhaled endotoxins is also believed to have an effect on airways resistance, presumably brought about by mediators which induce bronchoconstriction. The response is fundamentally different from the response in asthma in that it develops gradually over a period of about six hours. This pattern has been recognised among cotton mill workers for many years and it is colloquially referred to in Manchester as "monday morning fever" (Schilling et al., 1955). The symptoms are usually worse on monday morning following two days absence from work. Characteristically, the symptoms abort over subsequent working days and one is tempted to compare this with the phenomenon of experimental endotoxin tolerance.

4.2 THE NATURE OF BRONCHIECTASIS

4.2.1 Classification and Clinical Features

The observation that chronic expectoration of purulent sputum may be associated with a pathological dilatation of the bronchi is rightly attributed to the French physician, Laennec. This disease entity, now known as bronchiectasis, has a productive cough and haemoptysis as its major symptoms. Since these symptoms may occur in other respiratory disorders (especially chronic bronchitis) it may be difficult to differentiate from these other conditions. One helpful feature is that the cough may be precipitated by changes in posture and may be especially troublesome on first retiring at night or rising in the morning. Haemoptysis is common, usually slight in mild bronchiectasis but may be life threatening in advanced disease.

Physical signs are mainly those of bronchial secretions (mainly coarse crackles and wheezes) and are frequently widely dispersed through the lung fields. Cyanosis reflects the ventilation-perfusion mismatch consequent upon the lung damage. This is typically a feature of advanced disease in association with frank cor pulmonale. Finger clubbing is said to be very common and correlates with the degree of hypertrophy of the bronchial vessels.

The diagnosis is usually suggested by the history and physical signs. The only diagnostic test that is completely reliable is bronchography (Fig. 4.2). This is rarely performed nowadays except when the bronchiectasis is believed to be localised (e.g. right middle lobe bronchiectasis, also known as Brock's syndrome) and surgery is contemplated. A plain postero-anterior radiograph of the chest is frequently abnormal with visibly dilated bronchi and reticular mottling as the main findings.

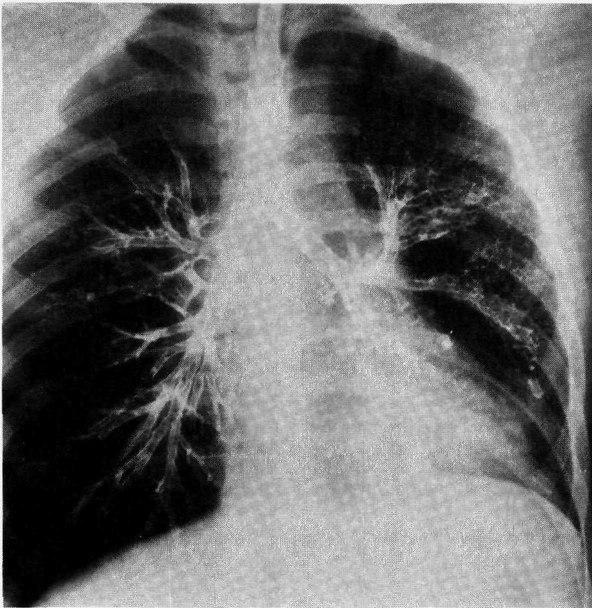

Fig. 4.2 Bronchogram from a teenager with localised left upper lobe bronchiectasis. Compare the normal right bronchial tree with Figure 4.1

4.2.2 Morphological Features of Bronchiectasis

Bronchiectasis may be separated into three forms on the basis of morphology, either determined by bronchography or at post-mortem examination (Table 4.1). Purulent secretions may be found within the dilated airways.

Microscopic examination reveals bronchial walls which have been damaged or completely destroyed (with only fibrous tissue remaining). Destruction extends to cartilage, muscle and elastic tissue. In saccular bronchiectasis the destruction may

TABLE 4.1 ANATOMICAL CLASSIFICATION OF BRONCHIECTASIS

Type	Description
Cylindrical	Dilated bronchi have regular outlines with 6-10 dilated branchings, often ending abruptly when obstructed by secretions.
Varicose	Bronchial walls are irregular with 2-10 branchings being visibly dilated. They are convoluted and manifest irregular constrictions.
Saccular	The size of the successive branches gradually increases, terminating in large sacs. Only 3-4 dilated branchings are usually visible. This form of bronchiectasis carries the worst prognosis.

extend into the pulmonary parenchyma with associated interstitial fibrosis and lung shrinkage. The lymphoid tissues in the lung are morphologically "active" and frequent nodules and follicles can be seen. A diffuse infiltrate of lymphocytes and plasma cells is often seen in the interstitium and some authors consider that these infiltrates precede the interstitial damage.

4.2.3 Pathogenesis of Bronchiectasis

This is best discussed in the context of an aetiological classification of bronchiectasis (Table 4.2). It must be clearly stated that no common mechanism for the bronchial destruction has yet been described.

TABLE 4.2 AETIOLOGICAL CLASSIFICATION OF BRONCHIECTASIS

Post-infective
Obstructive
Cystic fibrosis
Broncho-pulmonary aspergillosis
Primary ciliary dyskinesias (Kartagener's syndrome)
Immunodeficiency (Immunoglobulin deficiency; Chandra, 1983)
Cryptogenic

Post-infective bronchiectasis typically follows a childhood infection (measles, pertussis, scarlet fever) but also tuberculosis and pneumonia at any age. This form of bronchiectasis is declining as a result of improved public health, vaccination and anti-microbial chemotherapy.

Obstructive bronchiectasis is still encountered, often associated with thoracic neoplasms. One form of localised bronchiectasis which is sometimes encountered is Brock's syndrome (right middle lobe bronchiectasis). This is due to compression of the right middle lobe bronchus by enlarged lymph nodes of the primary tuberculous complex with a consequent retention of secretions. As the infection resolves, drainage is re-established but the lung is damaged.

Apart from cryptogenic bronchiectasis, the other causes of bronchiectasis alluded to in Table 4.2 are rare and since none of the patients recruited into our studies fell into these groups, they will not be discussed further. The term cryptogenic merely implies that no cause is known. This group is certainly not homogeneous. During the course of our studies we identified a number of patients with mild-moderate bronchiectasis (usually young or middle aged women) whose disease remained relatively stable and did not progress markedly over long periods of time. We also found a number of patients (usually older men) whose bronchiectasis was of comparatively recent onset and was rapidly progressive.

Whether the bacterial colonists contribute to the lung damage or not is, at present, unknown. However, Cole (1984) postulates a "vicious circle" hypothesis in cryptogenic bronchiectasis. After the initial damaging event the lungs are colonised by apparantly avirulent bacteria (e.g. Haemophilus influenzae), leading to further low-grade lung damage, sustained colonisation, and so on. He argues that normal defences are compromised and foreign materials are inefficiently cleared from the broncho-pulmonary tree. The attendant cellular response may indeed also contribute to the lung injury. One enzyme that has received considerable attention in this regard of late is elastase (Twumasi & Liener, 1977; Stockley & Burnett, 1979; Jackson et al., 1984). These studies reveal that elastase is frequently present in purulent sputum and that it is capable of damaging the bronchial epithelium. Furthermore, this low grade inflammatory response induces systemic changes in that it is associated with increased levels of C-reactive protein and amyloid A component (De Beer et al., 1982).

4.3 IMMUNOLOGICAL ASPECTS OF BRONCHIECTASIS

The published literature on this topic is very small and therefore easy to review. The first report of immunological abnormalities in bronchiectasis (Hilton & Doyle, 1978) came in the wake of reports of immunological abnormalities in a variety of respiratory disorders: alterations in the levels of circulating immunoglobulins in chronic bronchitis with emphysema (Biegel & Krumholz, 1968) and in cryptogenic fibrosing alveolitis (Hobbs & Turner-Warwick, 1967); an increased

prevalence of autoantibodies in cryptogenic fibrosing alveolitis (Tomasi et al., 1962), pneumoconiosis (Turner-Warwick & Parkes, 1970) and chronic bronchitis (Bonomo et al., 1966).

Hilton and Doyle recruited patients with chronic bronchial suppuration whose clinical findings and chest radiographs were consistent with a diagnosis of bronchiectasis. Only eleven of their fifty three patients had actually undergone bronchography. The patients in this study can be classified as having cryptogenic bronchiectasis and the main findings are summarised in Table 4.3 and are compared with the findings of Cole (1984).

TABLE 4.3 IMMUNOLOGICAL CHANGES IN BRONCHIECTASIS

	A	B
Elevated immunoglobulin levels (polyclonal)	79%	74.3%
Rheumatoid factor positive	52%	20%
Anti-nuclear factor positive	10%	17%

A: Hilton & Doyle, 1978 (53 patients)
B: Cole, 1984 (210 patients)

The severity of disease was associated with the recorded immunoglobulin levels in Hilton and Doyle's report. Patients with severe disease tended to have elevations in IgG, IgA and IgM while those with mild disease usually had elevations in only one class. Forty one percent of patients had increased IgA, 40% increased IgG and 51% increased IgM. Twenty eight percent had elevations in two classes of antibody and 15% had elevations in three. Nineteen percent of Hilton and Doyle's patients had overt autoimmune disease (mainly pernicious anaemia, rheumatoid disease and Sjögren's syndrome) as did 12.4% of the subjects in Cole's study. Fifty patients with chronic bronchitis had similar patterns of autoantibodies and immunoglobulin changes, though less marked and less frequent. Thirty three patients with bronchial asthma had only minimal changes.

Further studies by Hilton and her colleagues revealed that circulating immune complexes were a frequent finding in bronchiectasis (up to 67% of patients). The precise characteristics of these complexes were not defined (Hilton et al., 1978; Hilton et al., 1979; Cooper et al., 1981). In this context, it is interesting to note that some patients display transient episodes of cutaneous vasculitis (with the histological appearance of Henoch-Schönlein purpura) in association with exacerbations of their lung disease (Hilton et al., 1984). One of the patients in their report was taken from the present study.

Atopy is not associated with cryptogenic bronchiectasis but <u>is</u> a feature of

bronchiectasis in cystic fibrosis (Murphy et al., 1984). This suggests that the obstructive airways abnormality reported in their patients without cystic fibrosis may not be analogous to that seen in bronchial asthma, even though it was acutely reversed by the administration of fenoterol (a β2 agonist).

In summary, the main changes in those parameters tested in bronchiectasis are a high frequency of certain autoantibodies and polyclonal elevations in immunoglobulin levels. One is struck by the similarity of these changes to those seen in chronic liver disease and also that these changes can be induced experimentally by endotoxin (see Chapter I). The dilated airways of patients with bronchiectasis contain secretions in which large numbers of bacteria can be seen, including gram negative organisms. It is therefore not unreasonable to suggest that endotoxin derived from the bacterial colonists may contribute to the immunological phenomena seen in bronchiectasis. The results of two studies will now be presented, though for convenience, both studies will be discussed together.

4.4 THE RELATIONSHIP BETWEEN ENDOTOXINS AND CHRONIC BRONCHIAL SUPPURATION ATTRIBUTED TO BRONCHIECTASIS*

4.4.1 Materials and Methods

Twenty-four patients with chronic bronchial suppuration attributed to bronchiectasis were studied, eight men (mean age 54.9 years, range 39-68) and 16 women (mean age 45.2 years, range 24-68). Subjects were chosen from the diagnostic index of the Department of Respiratory Medicine at Manchester Royal Infirmary and invited to participate in the study. Inclusion into the study required that a history of chronic sputum production be present together with compatible radiographic changes (reticular mottling and/or visibly dilated bronchi). No patient was taking glucocorticoids or non-steroidal anti-inflammatory agents and all patients were in clinical remission. Fifteen healthy medical students served as controls for the LAL test.

Specific enquiry was made about smoking history, precipitating illness, sputum characteristics, dyspnoea, autoimmune disease, family history and recent antimicrobial chemotherapy. Investigations** included:

Spirometry
Sputum microscopy and culture
Full blood count
Blood sedimentation rate (Westergren)
C-reactive protein
Endotoxin assay
Immunoglobulin levels

---

* performed in collaboration with B.C. Leahy, R.A. Fox, T.B. Stretton and M.R. Haeney (Horan et al., 1984)

** Investigations not described in detail here are fully described in Chapter 3.

Rheumatoid factor
Antinuclear factor (ANF)
Smooth muscle autoantibodies
Reticulin autoantibodies
Mitochondrial autoantibodies
Gastric parietal cell autoantibodies

Spirometry

This was performed on the same dry Vitalograph spirometer before and after administration of 160 µg of isoprenaline by metered dose inhaler.

Haematological investigations

Cell counts and indices were obtained from EDTA specimens using an electronic cell counter (Coulter Electronics). The sedimentation rate (ESR) was performed on citrated blood and expressed as millimetres in the first hour.

Sputum

Smears were prepared and examined by light microscopy after Gram staining. Cultures were made under both aerobic and anaerobic conditions.

4.4.2 Results

Clinical assessment

The clinical data are summarised in Table 4.4. Thirteen patients (54%) attributed their bronchiectasis to an acute illness in childhood (pneumonia, scarlet fever, pertussis, measles). In the one patient with amyloid disease, the diagnosis of amyloid was made on renal biopsy. No patient was known to suffer from any connective tissue disease. None had a relevant family history of disease.

TABLE 4.4 CLINICAL DATA ON 24 PATIENTS WITH BRONCHIECTASIS

	Number
Previously smoking	5
Precipitating illness	13
Sputum (ml/day)	
25-100	14
150-200	9
250-300	1
Clubbing	3
Joint symptoms	3
Cutaneous vasculitis	2
Glomerulonephritis	1
Amyloid	1

The haemoglobin level and indices were within the normal range in all patients. The ESR ranged from 5 to 40 mm in the first hour and did not correlate with the level of C-reactive protein. The white cell count was within the normal range in all but two patients and the elevations in these two were minimal.

Sixteen patients (66%) had evidence of an obstructive ventilatory defect ($FEV_1/FVC < 0.7$) and in none was this reversible acutely with inhaled isoprenaline. The mean FEV_1 for all patients was 1.57 litres (mean predicted 2.68) and the mean FVC was 2.54 litres (mean predicted 3.4). Four of the patients with evidence of airflow obstruction had been smokers, though no patient had smoked within 10 years of the study.

Twelve patients had received antibiotics within 6 months of the study and five within 2 weeks.

Immunological investigations

The C-reactive protein and immunoglobulin levels and the results of the endotoxin estimations are summarised in Table 4.5 and Figure 4.3. The autoantibody data appear in Tables 4.6 and 4.7. Table 4.8 summarises the relationships between these.

TABLE 4.5 SUMMARY OF C-REACTIVE PROTEIN (CRP), IMMUNOGLOBULIN AND ENDOTOXIN ESTIMATIONS IN 24 PATIENTS WITH BRONCHIECTASIS, AND 15 CONTROLS

		Patients	Controls
CRP	3 mg/ml	3	15
	3.1-9 mg/ml	19	0
	9.1-15 mg/ml	2	0
IgG elevated		4	-
IgA elevated		7	-
IgM elevated		1	-
2 Immunoglobulins elev.		2	-
3 Immunoglobulins elev.		0	-
Endotoxin	2 hours	0	0
(gellation	2-3 hours	4	0
time)	3-4 hours	4	0
	4-24 hours	4	0

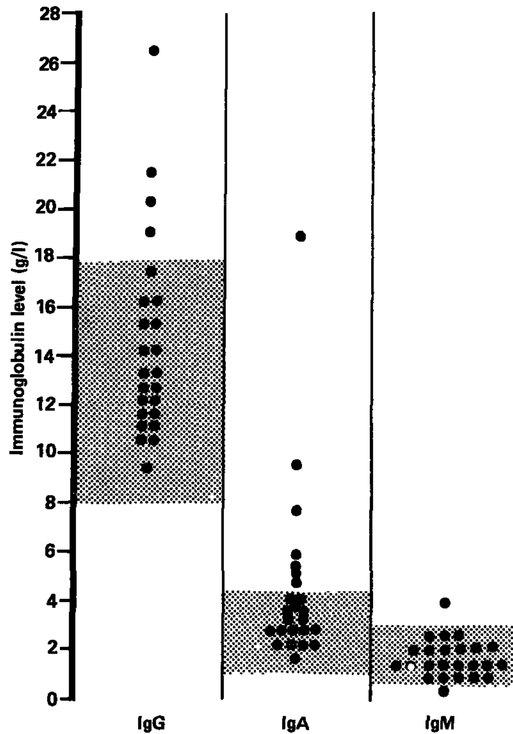

Fig.4.3 Results of serum immunoglobulin determination in 24 patients with bronchiectasis (shaded areas represent the reference range in adults up to the age of 65 and was derived in the hospital immunology laboratory).

For the patient population studied, the mean serum IgG was 16.5 g/litre (range 9.5-26.5), mean IgA 3.9 g/litre (range 1.75-19.0), and mean IgM 1.6 g/litre (range 0.35-4.1). No patient had elevations of all three classes of immunologlobulin. The patients with elevations in two, had a combination of IgG and IgA. No correlation was found between any of the immunological abnormalities described and previous antibiotic therapy.

TABLE 4.6 AUTOANTIBODY DETERMINATIONS IN 24 PATIENTS WITH BRONCHIECTASIS

Autoantibody	Number positive
Latex	
1/40-1/80	4
1/160- 1/320	3
SCAT	0
Smooth muscle	6
Reticulin	1
Mitochondria	0
Gastric parietal cell	4
Antinuclear factor	2

SCAT: Sheep Cell Agglutination Test
All autoantibody titres were low, but considered significant.

TABLE 4.7 AUTOANTIBODIES IN ENDOTOXIN POSITIVE INDIVIDUALS

Patient	Latex	ANF	GPC	RET	SMA
B.F.	+	-	+	-	-
M.B.	-	-	-	-	+
J.S.	+	-	-	-	+
S.K.	-	-	-	+	-
P.D.	-	-	-	-	+
H.P.	+	+	-	-	-
A.G.	+	-	-	-	-
D.G.	+	-	-	-	-
W.E.	+	-	-	-	+

ANF, antinuclear antibodies; GPC, gastric parietal cell autobodies; RET, antireticulin antibodies; SMA, antibodies to smooth muscle.
+ = positive
- = negative

TABLE 4.8 SUMMARY OF THE RELATIONSHIP BETWEEN IMMUNOGLOBULINS, AUTOANTIBODIES AND ENDOTOXIN IN 24 PATIENTS WITH BRONCHIECTASIS

	Endotoxin positive	Endotoxin negative
Ig + AA	6	0
Ig	3	2
AA	3	6
No Ig, no AA	0	4

Ig, immunoglobulin elevated.
AA, autoantibody present.

Bacteriology

The results are summarised in Table 4.9. One subject yielded two pure isolates. In 95% of patients gram-negative organisms were seen on microscopy of sputum.

TABLE 4.9 RESULTS OF AEROBIC* SPUTUM CULTURE FOR 24 PATIENTS WITH BRONCHIECTASIS

Organism	Numbers of isolates
E. coli	1
H. influenzae	13
Klebsiella spp.	2
N. meningitidis	1
S. pneumoniae	1
Proteus spp.	1
'Normal flora'	6

Sputum from one patient grew both S.pneumoniae and H.influenzae.

* No pathogens were revealed on anaerobic culture.

4.5 FURTHER STUDIES ON HUMORAL IMMUNITY IN CHRONIC BRONCHIAL SUPPURATION AND ASPECTS OF CELLULAR IMMUNITY

4.5.1 Materials and Methods

As for the study described in the previous section, subjects were chosen from the diagnostic index of the Department of Respiratory Medicine at the Manchester Royal Infirmary. After excluding subjects with only mild disease, 15 subjects were available for the study, five of whom had also participated in the previous study. Each subject was matched to a control of appropriate age and sex. In addition, pre-operative (elective) patients were used as further controls for spontaneous lymphocyte activation. These patients are reported on in Chapter VI. The following investigations were performed after a clinical assessment:

Immunoglobulin levels
Rheumatoid factor
Antinuclear factor (ANF)
Smooth muscle autoantibodies
Reticulin autoantibodies
Mitochondrial autoantibodies
Gastric parietal cell autoantibodies
T cell surface markers (immunofluorescence technique)
"Spontaneous" incorporation of tritiated thymidine
Autoradiography with detection of surface markers
Mitogen stimulation
Chromogenic Limulus Test
Sputum microscopy and culture

All blood samples were taken between 10.00 and 12.00 hrs.

<u>Mitogen stimulation</u>

Mononuclear cells were separated as described under Section 3.2.1. Cells were suspended in medium RPMI supplemented with 20% fetal calf serum and glutamine, at a concentration of 10^6/ml. Cells were then plated in microtitre plates at a density of 10^5/well together with an equal volume of medium RPMI containing the appropriate mitogen. The final concentrations used for Phytohaemagglutin (5 µg/ml), Concanavalin A (10 µg/ml) and pokeweed mitogen (5 µg/ml) were selected because they gave sub-optimal stimulation in normal adult controls from other experiments performed in our laboratory. After culture for 68 hrs at 37°C in humified air supplemented with 5% CO_2, 1 µCi of tritiated thymidine was added and the incubation allowed to continue for a further 4 hrs. Cells were then harvested onto glass fibre filters using an automated cell harvester. Thymidine incorporation was assessed in a liquid scintillation counter (LKB 81000) and, after subtraction of control counts, the results were expressed as Log_{10} counts per minute.

4.5.2 Results

Clinical assessment

The clinical data are summarised in Table 4.10. It will be noticed that these patients tended to produce more sputum and were more likely to possess finger clubbing than did the subjects from the previous study (Table 4.4).

The diagnosis of amyloid disease was made on renal biopsy in both cases.

TABLE 4.10 CLINICAL DATA ON 15 PATIENTS WITH MODERATE-SEVERE BRONCHIECTASIS

	Number
Previously smoking	2
Precipitating illness	9
Sputum (ml/day)	
25-100	6
150-200	4
250-300	3
350-400	2
Clubbing	10
Joint symptoms	3
Cutaneous vasculitis	2
Glomerulonephritis	1
Amyloid	2

Immunoglobulins and autoantibodies

The results of immunoglobulin determination are summarised in Figure 4.4. One striking difference from the previous study is that none of the patients had elevations in the IgM level. On the contrary, this parameter was depressed in five patients and a further patient gave a result which was just on the lower limit of normal. It must be pointed out that the lower limit of normal is somewhat high when compared with the published literature and many publications give a level of 0.4 g/l. Even so, three subjects would still be deemed to have low IgM levels. The relationship between the various immunoglobulin changes are summarised in Table 4.11. Table 4.12 summarises the autoantibody data. The subjects with benign monoclonal proliferations were both men in their early 60's. The concentrations of the homogeneous immunoglobulin component was less than 10 g/l in both cases and had disappeared on follow up several months later. On the basis of this limited information, it is impossible to accurately place these subjects into one of the categories of Radl's classification of monoclonal gammopathies (Radl, 1985).

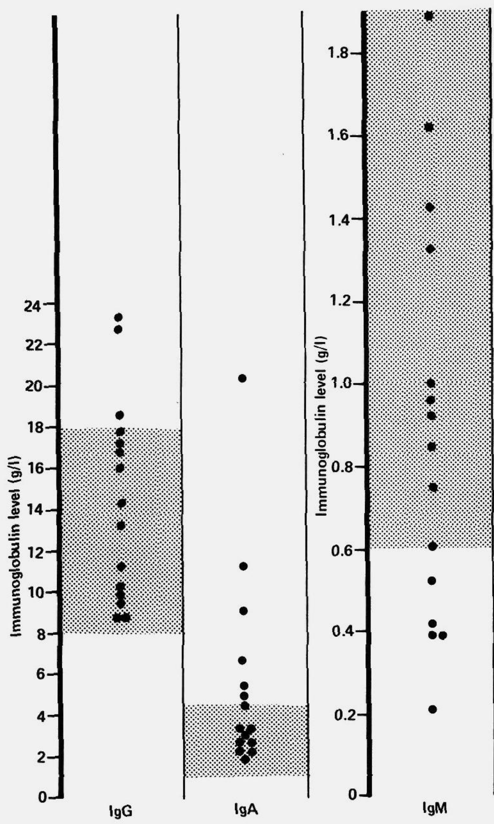

Fig.4.4 Results of serum immunoglobulin determination in 15 patients with bronchiectasis (shaded areas represent the normal range in adults).

TABLE 4.11 RELATIONSHIPS BETWEEN IMMUNOGLOBULIN ABNORMALITIES OF THE VARIOUS CLASSES IN 15 SUBJECTS WITH MODERATE-SEVERE BRONCHIECTASIS

	Patients	Controls
IgG elevated	3	0
IgA elevated	7 (2)*	0
IgM elevated	0	0
IgM depressed	5 (3)*	0
2 immunoglobulins elevated	3	0
IgM depressed, IgA elevated	2 (1)*	0
IgM depressed, IgG elevated	0	0
IgM depressed, IgA + IgG elevated	1 (1)*	0
Benign monoclonal proliferation (IgA)	1	0
Benign monoclonal proliferation (IgG)	1	0

* Figures in brackets refer to the number of subjects giving a positive Limulus test result.

TABLE 4.12 AUTOANTIBODIES DETECTED IN SUBJECTS FROM A GROUP OF 15 PEOPLE WITH MODERATE-SEVERE BRONCHIECTASIS

Antibody	Subjects	Controls
Latex		
1/40-1/80	5 (2)*	0
1/160	2	0
SCAT	0	0
Smooth muscle (G)	2 (1)*	0
Reticulin	2+	0
Mitochondria	1+	0
Gastric Parietal Cells	0	0
Anti-nuclear factor(IgG)	4+	0
Anti-nuclear factor(IgM)	6 (2)*+	0

* Figures in brackets refer to the number of subjects giving a positive result in the Limulus test.
+ = low titre.
G = glomerular and vessel pattern.

T lymphocyte subsets, mitogen stimulation and "spontaneous" lymphocyte activation.

The results of the membrane immunofluorescence studies (10 subjects) to ennumerate T lymphocytes and their subpopulations are summarised in Table 4.13. Significant differences (at the 5% level of probability) between subjects and controls (Wilcoxon's rank sum test for paired data) were found for the population of cells expressing the T8 antigen (suppressor/cytotoxic T cells) and for the ratio of T4 positive cells to T8 positive cells.

TABLE 4.13 MEAN PERCENTAGE OF CELLS EXPRESSING T CELL SURFACE MARKERS IN MONONUCLEAR CELL SUSPENSIONS FROM 10 PATIENTS WITH MODERATE-SEVERE BRONCHIECTASIS AND AGE/SEX MATCHED CONTROLS

		E. rosettes	T3	T4	T8*	T4/T8*
Patients	mean	72.6%	73.6%	43.3%	33.3%	1.2
	SD	(± 5.3)	(± 6.2)	(± 7.3)	(± 9.3)	(± 0.7)
	range	(69-86)	(67-84)	(33-56)	(20-52)	(0.72-2.67)
Controls	mean	75.0%	73.0%	50.0%	26.9%	1.9
	SD	(± 5.0)	(± 4.8)	(± 4.2)	(± 2.9)	(± 0.3)
	range	(70-85)	(65-82)	(44-59)	(23-34)	(1.5-2.55)

* indicates significant difference at 5% level of probability

TABLE 4.14 MEAN RESPONSE TO MITOGEN STIMULATION OF 10 PATIENTS WITH MILD-MODERATE BRONCHIECTASIS COMPARED WITH AGE/SEX MATCHED CONTROLS

	Phytohaemagglutinin (5 µg/ml)	Concanavalin A (10 µg/ml)	Pokeweed mitogen (5 µg/ml)
Patients	4.15	4.21	4.03
	(0.47)	(0.42)	(0.35)
Controls	4.8	4.5	4.39
	(0.28)	(0.33)	(0.25)

Results expressed as $\log_{10}$ counts per minutes.
Figures in brackets refer to standard deviations.

The results of the studies assessing the response to mitogen stimulation showed significant differences (Wilcoxon's rank sum test for paired data) at the 5%, *but not the 1% level of probability, with subjects showing diminished responses* for all three mitogens. These results are summarised in Table 4.14

In contradistinction to the slightly diminished (though significant at the 5% level) response to mitogens which was found in patients with bronchiectasis, "spontaneous" activation of lymphocytes was found to be increased. Since there was no overlap between the results from patients and those from controls, no further statistical analysis was necessary. These results are summarised in Figure 4.5. The relationship between "spontaneous" lymphocyte activation and measurement of endotoxin levels with the chromogenic limulus test is shown in Table 4.15.

TABLE 4.15 SPONTANEOUS LYMPHOCYTE ACTIVATION IN FIVE SUBJECTS WITH SYSTEMIC ENDOTOXAEMIA, AS ASSESSED BY A CHROMOGENIC LIMULUS TEST

Subject	Endotoxin Level (EU/ml)	Lymphocyte activation (counts per minute)
GM 160	0.21	1200
GM 198	0.32	800
GM 203	0.185	1000
GM 222	0.26	4400
GM 229	0.26	750

Sputum microscopy and culture

Sputum was examined for twelve of the fifteen patients and gram negative rods were observed in all specimens examined. A pure growth of Haemophilus influenzae was found in ten of the twelve sputum specimens while the remaining two grew E.coli and Proteus spp, respectively.

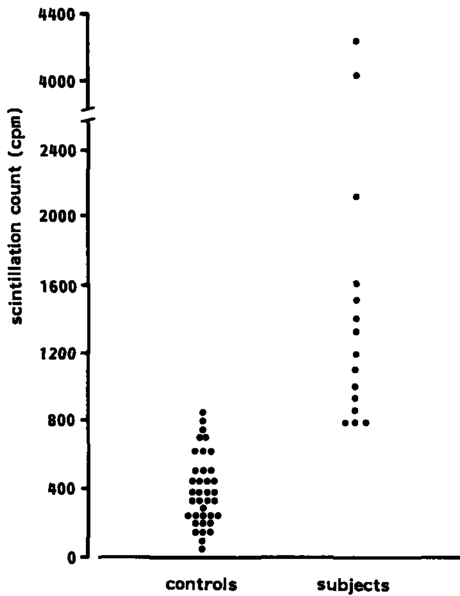

Fig. 4.5 Spontaneous lymphocyte activation in a group of 15 subjects with moderate-severe bronchiectasis and 37 controls (aged 30-50)

4.6 DISCUSSION

This chapter is chiefly concerned with two studies focussing on some immunological parameters in subjects with chronic bronchial suppuration attributed to bronchiectasis. Of the 34 subjects studied, 20 attributed their present pulmonary disease to an acute, severe respiratory tract infection in childhood. Two subjects with moderate-severe disease had recurrent episodes of cutaneous vasculitis (which displayed the histological features of Henoch-Schönlein purpura). These episodes of cutaneous vasculitis were associated with exacerbations of the pulmonary disease. Both of these subjects had persistent evidence of circulating immune complexes in the peripheral blood and the titres increased during the episodes of vasculitis (data not shown). Furthermore, all subjects from the second study were tested for evidence of circulating immune complexes and a positive result was obtained in 10 (67%); a figure in substantial agreement with that reported by Hilton et al. (1979).

Two subjects had evidence of amyloid disease which was confirmed by renal biopsy. Amyloid disease can be induced in mice by repeated intraperitoneal administration of endotoxin (Husebeek et al., 1985). It is proposed that the amyloid A protein derives from incomplete degradation of amyloid-related serum protein (SAA), an acute phase protein which can be induced by endotoxin (Pepys & Baltz,

1983). Normal human peripheral blood monocytes can completely degrade SAA but monocytes from patients with amyloid disease do not, and amyloid A protein can be shown to be generated in vitro by these cells (Fuks & Zucker-Franklin, 1985). Evidence that other mononuclear phagocytes may also be involved comes from studies on C57BL/6 mice in which amyloid was induced by the repeated administration of casein. Fuks and Zucker-Franklin (1985) showed that incomplete degradation of SAA by cultured Kupffer cells always preceded the development of amyloid disease. Reticuloendothelial capacity has not been assessed in patients with bronchiectasis.

SAA circulates complexed to high density lipoprotein (HDL) as an apoprotein (apo SAA)(Skogen et al., 1979). This observation is of particular interest since most of the circulating endotoxin is also bound to HDL. One hypothesis might be that endotoxin targets the HDL to mononuclear phagocytes and may also impair their ability to completely degrade SAA. The subject of HDL-endotoxin interactions is discussed in more detail in Chapter VII. De Beer (1982) has demonstrated increased levels of both C-reactive protein (as in this study) and SAA in subjects with bronchiectasis in the absence of amyloid disease.

Airflow obstruction was a very prominent feature of the subjects in this study and it was not acutely reversible by inhaled β-2 agonists. This suggests the mechanism may be fundamentally different from the airflow obstruction seen in asthma. It must be remembered that Murphy et al. (1984) reported atopy in subjects with bronchiectasis complicating cystic fibrosis and also that fenoterol reversed airflow obstruction in cryptogenic bronchiectasis. Recent work has shown that endotoxin can enhance IgE-mediated histamine release from human basophils (Smith et al., 1985). Very large numbers of mast cells occur in the human lung, both within and below the epithelium. It is already known that endotoxin can induce an increase in airways resistance, presumably as a result of active mediator substances. Endotoxin also promotes neutrophil migration into the airways and endotoxin can elicit leukotriene release from cultured human neutrophils. The magnitude of the leukotriene response shows considerable inter-individual variation (Brenn et al., 1984). Leukotrienes C4, D4 and E4 are very potent stimuli for airways smooth muscle contraction in man with the peripheral airways being much more sensitive than the central ones (Hammarström, 1983).

It is clear from the results of the microbiological investigations that the sputum of almost all subjects was sustaining the growth of gram negative bacteria at the time of testing. The dominant bacterial species was found to be the low-grade pathogen, Haemophilus influenzae. This is consistent with the findings of Cole (1984) and fits in with his notion of such low grade pathogens contributing in some way to the pulmonary damage which in turn impairs the host's ability to clear bacteria from the airways, thus forming a self-sustaining cycle ("vicious circle"). Figure 4.6 attempts to depict this notion as well as trying to suggest possible mechanisms.

So far, it has been argued that pulmonary gram negative bacteria and their products may explain (at least in part) the development of amyloid disease and airflow obstruction as well as forming part of a "vicious circle" which helps sustain the disease, but could they also play a role in the pathogenesis of other phenomena which can be observed in bronchiectasis? Perhaps one of the least controversial possibilities relates to some of the histological findings. Pulmonary lymphoid tissues are morphologically "active" and frequent nodules and follicles can be seen. Furthermore, a diffuse infiltrate of lymphocytes and plasma cells is often observed in the pulmonary interstitium. It seems not unreasonable that bacteria and their products could be directly responsible for these changes. If it is accepted that these bacteria may induce morphological evidence of stimulation of immunologically active cells, they would also be expected to affect their function. This possibility must be viewed in the light of the known immunological properties of endotoxins which are summarised in Chapter I.

The previously published literature (reviewed in the introduction to this chapter) clearly demonstrates that polyclonal immunoglobulin production and a high frequency of circulating autoantibodies are major immunological features of bronchiectasis. This is confirmed in this study. Such polyclonal elevations might even be anticipated in view of the large bacterial burden and the pulmonary tissue damage. The autoantibodies might result from the polyclonal stimulation or from some interaction between bacterial products and the products of the pulmonary parenchymal damage. The range of autoantibodies tested in this study is greater than previously reported. It would clearly be very useful to know whether antibodies were also being produced which recognise some substance to which the host has not been previously exposed. This would lend weight to the notion that endotoxin is inducing autoantibodies by activating lymphocytes rather than their production resulting from some kind of adjuvant effect.

Antibodies directed against smooth muscle were more common in subjects with mild-moderate disease (25% of subjects) while ANF was most common in those with moderate-severe disease (67% of subjects). One previously unreported (and as yet unexplained!) feature of severe bronchiectasis is a depressed level of IgM. Polyclonal elevations in immunoglobulin levels were somewhat more common in patients with moderate-severe disease and benign monoclonal proliferations were seen only in this group.

Prior to the studies reported here, cellular aspects of immunity had not been investigated. This study revealed a significant excess of T lymphocytes expressing the T8 surface antigen (cytotoxic/suppressor cells). Whether these cells were generated directly by endotoxin or formed part of a "down-regulating" physiological response was not established, but both possibilities are feasible. One very striking feature of patients with moderate-severe bronchiectasis is the excess of spontaneously active lymphocytes (which were presumably activated in vivo).

Endotoxin was detected in the peripheral blood in a minority of the subjects

studied but was never present in the absence of immunological aberrations. This suggests that systemic endotoxaemia is not a pre-requisite for the development of the immunological changes, and there was no direct correlation between the endotoxin level and lymphocyte activation. However, this finding is not crucial to the argument. It has already been pointed out (Chapter I, section 1.2.3) that prolonged culture in vitro is needed to induce proliferation of human lymphocytes. The current level of lymphocyte activation in vivo may well result from events at some time distant in the past. Furthermore, even this is not crucial to the argument. The ectatic airways support considerable numbers of bacteria and both they and their products might be anticipated to interact with immunologically competent cells within the lung itself. Regardless of whether the interactions occur locally or systemically, it still seems highly likely (in the light of their known properties) that endotoxins are responsible (at least in part) for the immunological aspects of bronchiectasis.

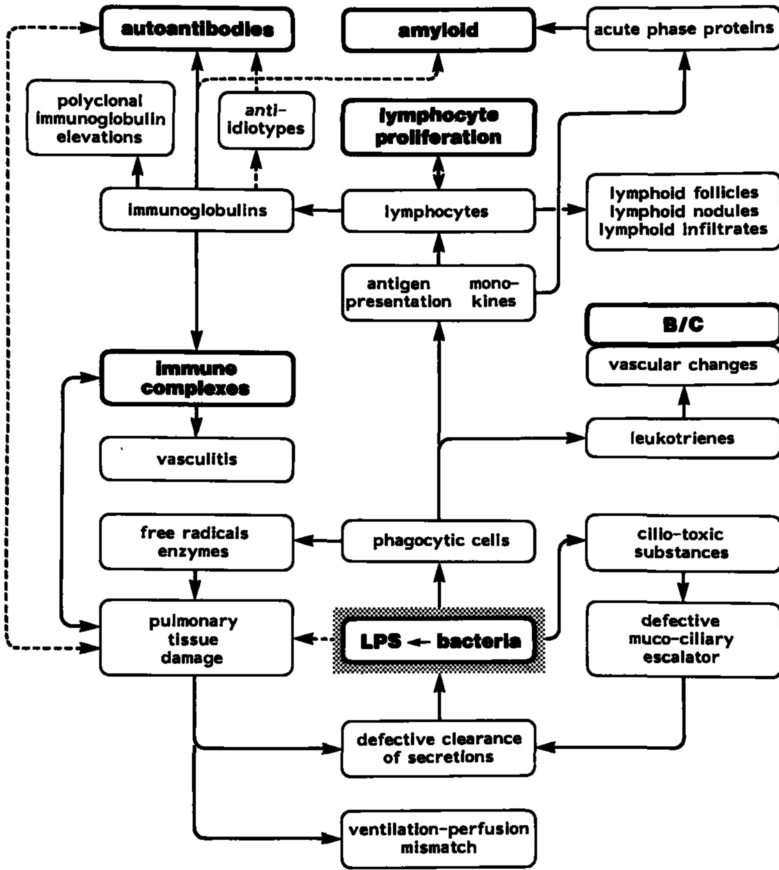

Fig.4.6 Hypothetical integrated scheme linking the pathogenesis of bronchiectasis with some clinical manifestations. The starting point is the box, LPS-BACTERIA. Following arrows in the bottom part of the figure, a number of pathways are proposed which are "self-sustaining". Following arrows upwards, the pathogenesis of the immunological changes, amyloid disease and bronchoconstriction is outlined.

B/C = bronchoconstriction

CHAPTER V

AGEING AND IMMUNE FUNCTION

5.1 INTRODUCTION

> "Ageing is by definition a highly heterogeneous phenomenon affecting every tissue to varying degrees, so that any attempt to depict its features must inevitably use a broad brush."
>
> Wright & Whalley, 1984

It is a truism to state that most (if not all) organisms enjoy a finite lifespan, though, in the natural state, very few organisms actually achieve it. In some species, the end of this period is signalled by some common cataclysmic event (e.g., steroid toxicosis in salmon). No comparable event has been identified in mammals (with the possible exception of "big bang" marsupials), though the observation of Ebbesen et al. (1982) that dying in aged mice (at least in C57BL and CBA mice) is associated with intense immunological activity and the generation of auto- reactive lymphocytes, is of interest.

One remarkably constant thread is that the <u>maximal</u> life expectancy within a species (or inbred strain) is relatively fixed. Furthermore, progression through this lifespan involves a number of comparable changes between species, designated by Finch (1976) as:

Post-natal development
Maturity
Middle Age
Senescence

This has been interpreted to mean that the appearance of age-related processes is not a random phenomenon, but rather an orderly progression which is probably genetically determined, though this does not necessarily imply that there must be an "ageing" gene. Although this concept remains unproven, a large body of circumstantial evidence has accumulated which broadly supports this notion.

It is widely accepted that such evidence is best explained by the presence of a very small number of genes which influence longevity and that these are closely associated with the major histocompatibility complex (MHC) (for review, see Popp & Popp, 1981). The MHC systems of the mouse and the human are the most completely understood and because of the multiplicity of MHC-related functions, it is often referred to as a "supergene".*

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* "closely linked genes controlling functions that are at least to some extent interrelated, and with respect to which in some cases, selective interaction occurs in such a way as to favour close linkage" (Bodmer, 1978).

The precise mechanisms of the process(es) of ageing have been the subject of great interest for a good many years. Unfortunately, no universally acceptable definition has emerged. A simple correlation between chronological age and some property or process is insufficient to establish it as a fundamental cause of functional decline with time. This dilemma led Strehler (1982) to propose four conditions which should be satisfied before any property or process can be designated as ageing, per se:

1) It should be DELETERIOUS
2) It should be PROGRESSIVE
3) It should be INTRINSIC
4) It should be UNIVERSAL

As Knook (1982) points out, any complex organism functions at several levels of organisation (molecules, cells, tissues, organs, etc.), all of which are inter-related, and that changes at any one level may profoundly influence function at another. In other words, ageing occurs in a dynamic setting and that for any given change, there is likely to be one or more secondary changes, and so on. By implication, the random descriptive approach will throw up observations which are secondary, and thus cloud the issue when what one is interested in is the nature of the primary phenomenon. The relatively minor nature of such changes when viewed in isolation may belie their true significance within a complex, integrated system. The alternative approach, that of systematically testing specific hypotheses is still in its infancy and has, as yet, not met with any great measure of success. One interesting hypothesis being pursued by Vijg and co-workers in this Institute is particularly attractive since it is amenable to such systematic testing and is also appealing in that it attempts to look at ageing in evolutionary terms.
It is argued that ageing does not make sense in evolutionary terms and that it is of virtually no relevance to organisms living in their natural state. Most (if not all) such organisms will die from other causes long before the adverse effects of ageing can make their presence felt (Fig. 5.1). Ageing may better be viewed as a continuation of processes which are critical during the developmental stage of an organism. DNA rearrangement, controlled by certain DNA processing systems, may be an integral part of normal development. Since DNA in the living cell is intrinsically unstable and continuously subjected to damage, an overlapping category of DNA processing systems (collectively termed DNA repair) has evolved whose function it is to correct damage which has been induced in the DNA. Many characteristics of DNA processing systems appear to be species-specific and their accuracy may well be related to the likely duration of survival for members of a species living in a "natural" environment. Species whose members tend not to to survive for prolonged periods of time (because of environmental hazards) do not require DNA processing systems which will sustain the integrity of their genome for, say, a hundred years. When environmental hazards are minimised (as is the

case in captivity), the effects of inaccurate DNA processing systems would slowly become manifest as DNA damage-induced illegitimate reactivation of differentiation-related rearrangement. The rate of such an hypothetical process would depend on the degree of inaccuracy of these DNA processing systems in that individual or species. If this notion is correct, it is not a great intellectual leap to accept that some of these manifestations are what we currently consider to be diseases. Ageing is inextricably bound up with disease. The elimination of such a disease process would not be expected to make the survival curve more rectangular but would simply shift it along the time axis. Another "disease" would simply be allowed to develop. Furthermore, it follows that some of the "diseases" which are associated with old age cannot be considered as separate from ageing. These speculations are not merely philosphical in nature but have very real implications for designing experiments on ageing in old humans.

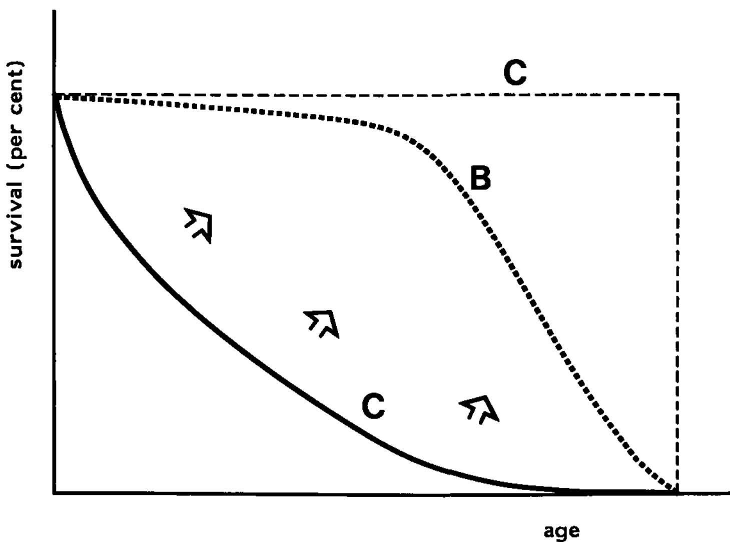


Fig.5.1 Shape of the survival curve.

Curve A represents an ideal rectangular curve in which animals enjoy full vigour until some pre-determined time and then all members of that species or strain rapidly die.
Curve B approximates to the shape of the current human survival curve.
Curve C approximates to the shape of the survival curve in animals living in the wild.

In the light of the above, the following discussion on "immunogerontology" must therefore take place in the absence of any precise definition of what ageing is; whether it is manifested as a single primary process or multiple ones; and whether it is manifested in precisely the same way in all species.

5.2 THE IMMUNE SYSTEM

5.2.1 Introduction

Many changes have been described which are in no way universal and their intrinsic, progressive and deleterious nature not established. Some of these changes are probably artefacts of the design of the studies. One change that has attracted considerable attention is involution of the thymus gland and this will now be considered in some detail after discussing changes in the bone marrow.

5.2.2 Bone Marrow

All haemopoietic cells derive from a common pluripotent stem cell in the bone marrow. Micklem and Ansell (1984) found no defect in the stem cell compartment in ageing CBA mice. Animals were exposed to hydroxyurea at 3-4 week intervals throughout adult life and were killed 5-9 weeks after their final dose. No stem cell defect was identified, either in terms of spleen colony forming cell numbers (CFU-S) or in terms of the ability to compete with marrow cells from young animals in repopulating lethally irradiated recipients. In a recent review, Wade & Szewczuk (1984) were able to state that the bone marrow of aged mice fully retains the potential to produce cells with a normal available and functional immune repertoire once removed from the aged environment. Within the spleen, there are changes in the distribution of cells as a function of age and the cells that are present can individually display full cell function. They went on to conclude that the available repertoire of specificities, idiotypes and V region genes within the bone marrow is functionally indistinguishable between young and old mice and that any age-related modification occurs during the selection of the functional repertoire.

5.2.3 Thymus

Development along the different differentiation pathways appears to be induced by specific thymic micro-environmental events. For T lymphocytes, the major inductive influence is the thymus gland (reviewed by Weissman et al., 1982). The progenitors of T cells (prothymocytes) migrate from the bone marrow via the blood and are first located in the subcapsular cortex. These cells lack typical T cell differentiation antigens, but give rise to cortical thymocytes (which together constitute the major cell population of the thymus gland). These cells do express characteristic T cell differentiation antigens such as T4 and T8 (in man), the thymus cortex specific antigen (T6) and the enzyme, terminal deoxynucleotidyl

transferase (TdT). Kay (1984) reported that movement of precursors to the thymus ceases prior to adolescence in long-lived, autoimmune disease-resistant mice, as assessed by chromosomal markers in parabiotic mice. She suggested that some extra-thymic regulatory mechanism may be operating since the precursor cells did indeed retain their capacity to migrate to a thymus and that even an involuting thymic graft was capable of accepting them. Steinmann and Müller-Hermelink (1984) reported that TdT was continuously present in a very large number of human cortical thymocytes throughout life and suggested that the human thymus can (and may well) preserve a microenvironment that can support cells producing TdT and that the gland may continue to play a physiological role throughout life.

With further development, T6 and TdT gradually disappear and two distinct populations emerge which can be distinguished as T1, 3 and 4 positive and T1, 3 and 5/8 positive. Studies utilising human peripheral blood T lymphocytes suggest that T4 positive cells are associated with helper activity while T5/8 positive cells are associated with suppressor/cytotoxic activity. These lymphocytes have also become MHC-restricted in that antigens are only recognised in the context of Class I (HLA-A,B,C) or Class II (HLA-DR, Dc, SB) antigens of the major histo-compatibility complex. For cytotoxic T cells, the relevant MHC antigens are Class I (present on all cells). For T helper cells, Class II molecules are required (present in large numbers only on antigen presenting cells). Suppressor T cells are heterogeneous in that some appear to recognise foreign antigens in the context of Class I molecules, some in the context of Class II and some can probably do so directly.

Studies by Steinmann and Müller-Hermelink (1984) suggest that the human thymus can support T cell differentiation and generate new T cell clones throughout life. They used thymic biopsies and followed the distribution of both MHC antigens and T cell differentiation antigens. This revealed that immature T cell precursors occur throughout life, though there was an increase in medullary T cells expressing the surface markers of cytotoxic/ suppressor cells. They also demonstrated areas packed with immature T cells which were without contact with "educating" epithelial cells. Functional studies by the same authors (Steinmann & Müller-Hermelink, 1984) showed that human thymocyte suspensions showed an increased response to the mitogen concanavalin A (ConA) and the lymphokine interleukin 2 during ageing. There was an enhanced production of interleukin 2 (IL-2) in stimulated thymocyte suspensions and an increase in IL-2 responsive cells in the thymic cortex. Converse changes as a function of age were observed in spleen cell suspensions and in peripheral blood. Oosterom and Kater (1981) suggested that the increased responsiveness of human thymocytes to mitogens was due to a relative increase of the more mature, medullary thymocytes since there is no change in the proportion of macrophages and a decrease in the number of cells responsive to HTECM (human thymic epithelial-conditioned medium), which enhances the mitogen responses of thymocytes from young donors. These pheno-

mena were not observed in thymocyte suspensions from rodents.

Many studies have reported that the human thymus gland becomes smaller with advancing age (reviewed by Kendall, 1981). Most of the glands studied (especially from older subjects) were obtained post-mortem. This is rather unfortunate since it is now known that the thymus may change dramatically over a period of days as a result of a number of stresses (disease, surgery, pregnancy, endocrine changes etc.; Kendall, 1981). More recent studies have demonstrated a considerable variation in the size of the thymus, particularly in old age. For example, the range in man in the seventh decade was 1-55 grammes. The composition of the thymus undergoes more consistent age-related changes. Fat accounts for an ever increasing proportion of the gland between 20 and 50 years, but does not tend to greatly increase thereafter. Notwithstanding, the human thymus certainly maintains an (morphologically) active cellular compartment, even into advanced age. Furthermore, the size of this compartment is greater in biopsy specimens than those obtained post-mortem. Just as exogenous factors influence the size of the gland, they may also influence its composition. In the fox (Vulpes vulpes), during periods of weight loss, the thymus loses its lobulation and is subject to intense lipid deposition with almost complete regression of the lymphoid cellular mass. Recovery after this period is rapid, with a return of lobulation and a restoration of the cellular mass, so that the gland comes to resemble that of juvenile foxes.

A number of studies in mice have shown that sequential grafting of syngeneic neonatal thymus glands enhances T cell dependent immune functions, if the grafting procedures commence while the animal is young (e.g. Hirokawa et al., 1982; Hirokawa & Utsuyama, 1984). When such grafting commences in "middle age" (17 months in C57BL/6 mice) there was no restoration of the mixed lymphocyte reaction and the response to sheep red blood cells and only partial restoration of the response to the mitogen phytohaemagglutinin. Co-transplantation of bone marrow corrected this deficiency. Such experiments in laboratory rodents should be interpreted with great care. There is certainly evidence that age-dependent changes in the mouse thymus and young adult thymectomy have marked effects on peripheral T cell functions (reviewed by Van de Griend et al., 1982). There is still but little reliable information on the long term sequelae of adult thymectomy in man. Van de Griend et al. (1982) report that adult thymectomy for non-immunological disease in adult humans produces relatively minor changes in human peripheral blood lymphocytes and (in contrast to what happens in mice) they conclude that the thymus may play only a minor role in the maintenance of immune competence in the adult human. This finding is interesting in the light of an unpublished study from the TNO Institute for Experimental Gerontology in which neonatally thymectomised rats (SPF-derived) were maintained in "conventional" conditions and failed to show any impairment in survival or any increase in the frequency of infections (Hollander, Zurcher & Boorman 1976, unpublished results).

The thymus gland does not function solely as a site for T cell development but is a site of haemopoiesis and is known to secrete a number of humoral factors which may act both locally and systemically and influence both T cell development and function. Thymopoietin activity of human serum is well maintained up to around 30 years of age but is undetectable by the age of 60. Thymulin (previously known as Facteur Thymique Sérique, FTS) falls after the age of 20 and is undetectable by 50. Thymosin α-1 declines even earlier. Thymulin is absolutely dependent upon zinc (which on binding, induces a conformational change) both for its biological activity and immunogenicity. Dardenne et al. (1984) studied thymulin production in a number of mouse strains and confirmed that thymulin activity is low in aged animals as it is in young representatives of autoimmune-prone strains. They also demonstrated the presence of low molecular weight inhibitors in human blood after about 30 years of age and that addition of zinc in vitro did not restore thymulin activity in this setting. It is also clear that plasma levels of thymulin are profoundly influenced by other hormones. For example, thyroxine markedly enhances thymulin production while low levels of thyroxine suppresses them (Fabris et al., 1984). These authors were led to propose that thymulin production is not autonomous and that derangements in some "non-primary immunodeficiency disorders" are not necessarily irreversible phenomena. Thymulin production can be suppressed in protein/calorie malnutrition (not uncommon in aged humans) with enhanced production with nutritional supplementation (Chandra et al., 1982). Some studies have shown that administration of thymosin α1 can enhance T helper functions in aged mice and that this is associated with enhanced production of, and responsiveness to, interleukin-2 (Frasca et al., 1984).

5.2.4 T Lymphocytes

The statement "there is an overall loss of T cell functions with advancing age" is frequently encountered in the immunogerontology literature. It must always be remembered that T cell responses involve complex cellular interactions and the demonstration of a deficit does not necessarily imply that a particular subset is intrinsically at fault. In vitro lymphocyte transformation in response to phytohaemagglutinin (PHA), concanavalin A (ConA) and allogeneic target cells declines with age in most species studied (Mathies et al., 1973; Gerber & Braun, 1974; Fernandez et al., 1976; Goodwin et al., 1982). It must however be emphasised that there are occasional papers which give contrary results. The impaired response to PHA is believed to represent an intrinsic T cell defect in that it does not reside in the accessory cells. Only a proportion of T lymphocytes are responsive to PHA and in mice they can be distinguished from T helper and T suppressor cells, both by surface markers and by function. The functional significance of this cell population remains to be determined. The cell populations responsive to ConA are different from the PHA responsive cells and include T suppressor cells. Möller has recently drawn attention to a disparity between in vitro and in vivo activities of

concanavalin A-activated lymphocytes. In his study (Möller, 1985), concanavalin A activated T lymphocytes displayed suppressor activity in vitro but could display helper activity when injected into athymic (nu/nu) mice. Evidence was presented that the mechanism of in vitro suppression is by absorbing a necessary growth factor (probably interleukin-2 (Palacios & Möller, 1981). Suppressor T lymphocytes are not a uniform population, some being specific and some non-specific. It is not known (but is believed by some authors) whether non-specific suppressor T cells are a completely separate population or are in fact precursors of specific suppressor T cells. Globerson (1984) raises the possibility that although these cells are not antigen-specific, they may be specific for idiotypic or allotypic determinants. This remains an open question. It will be recalled from Chapter I that the gut flora appears to play an important regulatory role in the generation of non-specific (promiscuous) suppressor T cells. Globerson (1984) reports an expansion of this non-specific suppressor T cell compartment with ageing in mice.

In man, most studies report either no change in the total number of T lymphocytes or that there is a decrease (reviewed in Kay & Makinodan, 1981). A recent study applying the formalised inclusion criteria of the Senieur protocol reported an increase in the proportion of cells expressing surface markers both of helper and suppressor T cells, but not of pan-T cell markers (Ligthart et al., 1985). This may mean that there is a loss of the T3 antigen or that cells co-express T4 and T8. Such co-expression has been recognised in young adults and seems to be a feature of activated lymphocytes (Blue et al., 1985). Perhaps the latter possibility is more likely since there was no increase in cells positive for surface markers of immaturity. Ligthart's study also showed a marked increase (of about 50%) in the numbers of non-T, non-B lymphocytes (null cells). Many of the cells in this compartment reacted with Leu 7 (which is thought to recognise natural killer (N.K.) cells, though not all Leu 7 positive cells were null cells. There was an increase in Leu 7 positive cells as a function of age.

Delayed-type hypersensitivity (DTH) as tested by responses to cutaneous injection of a battery of recall antigens (e.g., mumps, Candida, Trichophyton, streptokinase-streptodornase, purified protein derivative of M.tuberculosis) or by primary sensitisation with dinitro-chlorobenzene (DNCB) reveal a fairly consistent decline with age (reviewed by Makinodan & Kay, 1980). It must be remembered that DTH responses can be impaired by exogenous factors (such as nutritional status and trauma - see Chapter 6). For example, vitamin A deficiency is associated with profound suppression of DTH responses.

It has been suggested that one common thread which may explain the T cell functional deficits above might relate to production of, and responsiveness to, IL-2. It has been shown in rats, mice and humans that there is an age-related decline in the production of, and responsiveness to this substance (Thoman-Weigle, 1982; Gillis et al., 1981) However, such changes may be seen in some stressful situations such as surgery (see Chapter VI).

One piece of very convincing evidence that there is a truly intrinsic lymphocyte defect as a function of ageing comes from studies on isolated nuclei (Gutowski et al., 1984). PHA-stimulated lymphcytes contain a cytoplasmic factor (ADR) which stimulates the incorporation of tritiated thymidine into isolated nuclei. Peripheral blood lymphocytes from aged humans generate comparable amounts of ADR, but the nuclei from aged donors appeared to have problems in recognising this cytoplasmic signal for DNA replication.

5.2.5 Humoral Immunity (Reviewed in Kay & Makinodan, 1981)

Most studies report either no change or a slight decrease in the number of peripheral blood B lymphocytes with an increase in the spleen and lymph nodes of mice. The aggregated amount of circulating immunoglobulins (which must ultimately reflect the activity of the entire B cell compartment) shows no age-related change, though there is a tendency for an increase in IgG and IgA and a decrease in IgM. The levels of "natural antibodies" to blood group antigens and certain ubiquitous microbes, declines with age. Specific responses to exogenous antigens are less pronounced in old age, though adequate protection has been demonstrated with a number of vaccine preparations (Solomonova & Vizels, 1973; Feery, 1974; Bentley, 1981). It is suggested that the impairment in antibody production is partly secondary to a T cell disorder and partly intrinsic. Changes in membrane fluidity and cytoskeletal abnormalities have been demonstrated in rat B lymphocytes (Woda et al., 1979). In the face of these modest declines in antibody production stands one of the most important paradoxes in immunogerontology. Almost all studies (e.g. Batory et al., 1985; Goidl et al., 1981; Goodwin et al., 1982; Hijmans et al., 1984; Ockhuizen et al., 1982; Pandey et al., 1979) demonstrate an increased frequency and ease of induction of autoantibodies, especially to gastric parietal cells, thyroid epithelial cells and nuclear antigens, with advancing age. Furthermore, there is an increased frequency of polyclonal elevations in immunoglobulin levels and a dramatic increase in the frequency of benign monoclonal proliferations. Although these may all reflect some underlying disorder in immunoregulation, they may be equally well explained by the presence of exogenous immunomodulatory substances. It is with this latter possibility that the studies in section 5.3 are chiefly concerned.

5.2.6 Macrophages

These cells play an important role in the immune response, both in terms of presentation of antigen and in the production of regulatory molecules (monokines). The presence of macrophages may modify the B lymphocyte response to endotoxin (Veit & Feldman, 1976). Yoshinaga et al. (1972) have demonstrated an inhibitory effect by macrophages on the B cell proliferative response to endotoxin in mice. It is known that endotoxins induce macrophage prostaglandin synthesis and that some

prostaglandins are potent inhibitors of the B cell mitogen response. In this context it is of interest that peritoneal macrophages will suppress in vitro proliferation of spleen cells in most mouse strains. This property is lost in aged NZB mice and the macrophage will no longer suppress (Ibrahim et al., 1982). This strain develops autoimmune phenomena at a young age. Non autoimmune-prone strains retain this macrophage suppressor activity. Evidence exists that both antigen presentation (Effros & Walford, 1984) and interleukin-1 production (Inamizu et al., 1985) may be defective in old C57BL and BALB/c mice.

Other functions may not decline with age. For example, in vitro phagocytosis by mouse peritoneal macrophages is increased in old age(Perkins, 1971). It has also been shown that lysosomal enzyme activity is increased in the peritoneal macrophages of old mice compared with young (Heidrick, 1972; Platt & Pauli, 1972). In certain mouse strains such as NZB (which are prone to autoimmune phenomena), lysosomal enzyme activity is higher than in non-autoimmune-prone strains such as BALB/c (Bar-Eli & Gallily, 1979). The number of resident peritoneal macrophages also increases with age (Perkins, 1911).

It appears that macrophages are activated and mobilised in old age. This should be considered in conjunction with the reported loss of suppressor activity by macrophages of aged NZB mice. It is tempting to speculate that the two are related, suppression being lost by depletion of factors because of repeated stimulation. The fact that increased numbers of resident pesitoneal macrophages are to be found in old age is of great interest. It was suggested by Horan and Fox (1984) that this resulted from the presence of chemo-attractants, possibly endotoxin.

5.3 ENDOTOXINS AND "SPONTANEOUS" LYMPHOCYTE ACTIVATION IN AGED HUMANS

5.3.1 Introduction

Preliminary studies demonstrated increased "spontaneous" lymphocyte activation in elderly humans while yet other studies revealed circulating endotoxin activity in apparantly healthy subjects. It was therefore decided to determine if the two phenomena might be related. It was also decided to measure plasma fibronectin as a possible indicator of reticuloendothelial activity.

5.3.2 Materials and Methods

Forty individuals over the age of seventy were chosen from among those occupying beds administered by the Geriatrics Medical Service in Salford and were awaiting discharge. A further twelve were recruited from attenders at a health screening programme. All subjects were chosen because their medical problems were stable, there was no clinical evidence of infection or malignancy and were

taking no drugs known to influence immunological parameters. Young controls (n=37) between the ages of thirty and fifty comprised laboratory workers and patients admitted to hospital for elective surgical procedures. Any biochemical or microbiological investigations were performed as suggested by the clinical situation. The following specific investigations (described in Chapter 3) were performed:

Detection of T cell markers (immunocytochemical method);
Spontaneous incroporation of tritiated thymidine;
Identification of spontaneously active lymphocytes;
Augmented, chromogenic LAL test;
Fibronectin concentration.

5.3.3 Results

5.3.3.1 Lymphocyte characterisation

The results of the lymphocyte characterisation studies are shown in Table 5.1. No significant difference was detected between patients and controls with any of the monoclonal antibodies used. This suggests that there is no change in the total numbers of suppressor/cytotoxic T lymphocytes or in T helper lymphocytes.

TABLE 5.1 THE DISTRIBUTION OF PERIPHERAL BLOOD T LYMPHOCYTES AND THEIR SUBPOPULATIONS DETERMINED BY THE DETECTION OF THE T3, T4 AND T8 MEMBRANE ANTIGENS IN 52 SUBJECTS OVER 70 YEARS OLD AND IN 37 CONTROLS BETWEEN THE AGES OF 30 AND 50.

	Percentage of cells recognised by		
Subjects	OKT3+	OKT4+	OKT8+
Patients	77.3%	44.0%	31.7%
	(5.44)	(7.31)	(7.87)
Controls	75.7%	48.7%	27.3%
	(4.9)	(4.34)	(3.13)

* Figures in brackets represent standard deviation.

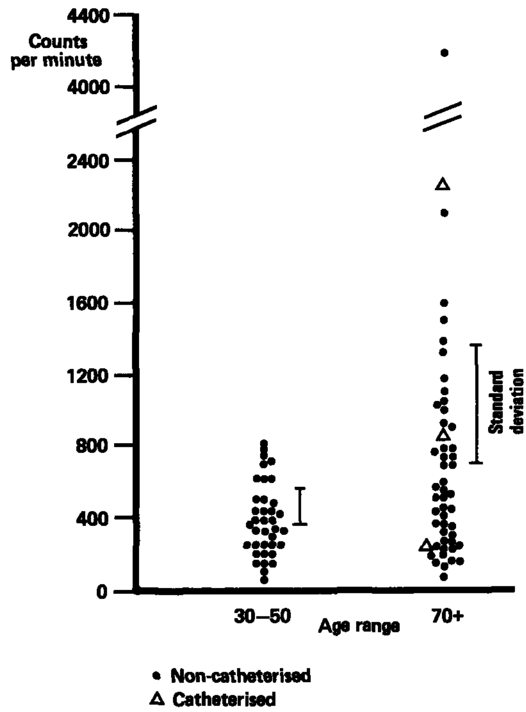


Fig.5.2 Spontaneous lymphocyte activation in 51 subjects over the age of 70 and 37 controls (aged 30-50) as assessed by liquid scintillation counting.

5.3.3.2 "Spontaneous" incorporation of tritiated thymidine

"Spontaneous" lymphocyte activation as assessed by thymidine incorporation over a two hour period is illustrated in Figure 5.2. It is clear from this figure that 37 of the aged individuals gave results which are similar to the results from the younger controls, though some old individuals gave results which are higher (some considerably so). Five of the old individuals also had a positive Limulus test (one of whom was subsequently demonstrated to have an E.coli urinary tract infection and 30 hours later developed a septicaemia). None of the younger controls had a positive Limulus test.

5.3.3.3 Fibronectin determinations

Fibronectin determinations for the five aged subjects with a positive Limulus test are shown individually in Table 5.2. In addition, plasma fibronectin levels (as an indicator of reticuloendothelial function) were determined in 44 of the aged subjects and in 26 of the younger controls. These results are summarised in Table 5.3. These levels were not normally distributed in either the aged subjects or the younger controls. The older subjects showed negative skewing about a median value of 33.32 mg/dl while the younger subjects showed a positive skewing around a median value of 22.37 mg/dl. Furthermore, much more variation in plasma fibronectin levels was found in the older subjects than was apparent in the young controls. There is also a significant increase in plasma fibronectin level (Mann-Whitney U test, 5% level of probability) with age.

TABLE 5.2 A SUMMARY OF THE POSITIVE ENDOTOXIN RESULTS RELATED TO THE RESULT OF SCINTILLATION COUNTING AND THE PRESENCE OR ABSENCE OF INFECTION

Patient	Endotoxin level	Scint count	Urinary Catheter	Inf	Blood culture positive	Fibronectin (mg/dl)
1	23 pg/ml	840 cpm	-	-	-	23.67
2	14 pg/ml	1360 cpm	-	-	-	18.62
3	18 pg/ml	1580 cpm	-	-	-	8.73
4	15 pg/ml	2230 cpm	-	-	-	17.08
5	42 pg/ml	4200 cpm	+	+	+	49.99

Scint count = scintillation count value of mononuclear cell suspension.
Inf = infected
+ = yes, - = no

TABLE 5.3 PLASMA FIBRONECTIN DETERMINATIONS IN 44 AGED INDIVIDUALS AND 26 YOUNGER CONTROLS

Parameter	Young controls	Aged Subjects
Fibronectin levels (mg/dl)		
- mean	23.116	31.659
- median	22.365	33.32
- SD	5.552	7.594
- range	19.73-38.99	18.73-45.99
- C.o.V.	.175	.329

C.o.V.= coefficient of variation

5.3.3.4 The nature of spontaneously active lymphocytes

This could be defined for only 13 subjects in the study (all from the aged group) since the remainder had such low levels of lymphocyte activation that accurate characterisation proved impossible. This means that the results summarised in Table 5.4 cannot be compared with the "normal" pattern of lymphocyte activation in young or old subjects with a scintillation count value in the range 200-800 cpm. It is, however, clear that a difference exists between the expression of the T3 antigen and the predicted expression of T4 and T8. The sum of T4 and T8 expression exceeded the T3 expression in 11 of the 13 subjects.

There are three possible explanations for this:

1 Inaccuracies in the method
2 Loss of T3
3 Co-expression of T4 and T8

It is unlikely that these results are an artefact of the methods used since such a discrepancy does not exist when total peripheral blood mononuclear cells are characterised with this immunocytochemical technique. Either loss of T3 or co-expression of T4 and T8 must be entertained as likely explanations.

TABLE 5.4 EXPRESSION OF T CELL SURFACE ANTIGENS ON "SPONTANEOUSLY" ACTIVE MONONUCLEAR CELLS* FROM 13 SUBJECTS OVER 70 YEARS OLD

Subject	Scintillation count	T3(%)	T4(%)	T8(%)	T3-(T4+T8)
MAH 68	4200	72	44	26	+2
MAH 41	2100	68	47	33	-12
MAH 16	1600	56	39	24	-7
MAH 128	1500	53	37	29	-13
MAH 112	1400	77	48	31	-2
MAH 50	1350	61	34	29	-2
MAH 47	1200	69	36	34	-1
MAH 83	1150	57	36	32	-11
MAH 69	1100	71	41	36	-6
MAH 146	1100	76	39	31	+6
MAH 101	1000	63	42	28	-7
MAH 96	950	59	33	33	-7
MAH 27	800	72	43	36	-7

* autoradiographically positive cells were counted.

5.4 DISCUSSION

The introduction to this chapter briefly reviewed the age-related changes in the immune system. The major paradox of immunogerontology was highlighted. In the face of the rather general (though minor) decline in antibody responsiveness to foreign antigens (non-self) there is an increase in various autoantibodies (self). Nowadays it is widely accepted that the potential to develop autoantibodies is universal (though not usually manifest) in the sense that the repertoire of T- and B-lymphocyte receptors includes not only those which can recognise allo- and xeno- but also self-determinants. The major unanswered question is not so much how "self-reactive" clones arise but how they are normally suppressed or eliminated. Evidence exists in support of various mechanisms which provide only partially satisfactory explanations for self-tolerance:

1) Anatomical segregation from the immune system.
2) Clonal elimination or anergy of B-cells induced by antigen (or by anti-Ig) during maturation to the stage when they first express specific receptors.

3) Suppression of immunoglobulin secretion by mature B-cells due to interaction with antigen (high concentration or prolonged) in the absence of T-cell help.
4) Absence of T-cell help, either because potential T-cells are themselves made tolerant or because the antigen is not presented in such a way as to elicit T cell help.
5) Generation of suppressor T-cells which specifically suppress B-cells.
6) Generation of suppressor T-cells which suppress T-helper cells.

Breakdown in or circumvention of these mechanisms could result in autoantibody production:

a) Loss of anatomical segregation caused by injury or infection.
b) Stimulation by an exogenous antigen which cross reacts with components of self which are normally non-immunogenic.
c) Stimulation of antibodies by an exogenous antigen or adjuvant which binds to self components.
d) Alteration of self components and thereby presentation of immunogenic novel configurations.
e) Activation of non-specific T-helper cells such as is thought to occur in graft versus host disease.
f) Polyclonal activation of B-lympohocytes.

A number of polyclonal B-cell activators might arise naturally in an intact animal from time to time. These include Staph. aureus protein A, Klebsiella pneumoniae polysaccharide, components of Mycoplasma pneumoniae and lipopolysaccharides from gram negative bacteria. The last of these possibilities is probably the most relevant.

The notion that polyclonal activation might explain the "paradox" of immunogerontology was first formulated by Cohen and Ziff (1977). They drew attention to two consequences of such a mechanism:

1) Increasing numbers of B-cell clones become committed to the production of antibodies, some of which are directed against "self".
2) This commitment leads to a decline in the number of B cell clones available for antibody production towards new antigens to which the ageing individual is exposed.

There is no doubt that polyclonal activators can give rise to autoantibody production, both _in vivo_ and _in vitro_. For example, numerous autoantibodies are seen in a variety of protozoan-induced diseases (Goodman & Weigle, 1982). Endotoxin can induce antibodies which are cytotoxic for syngeneic spleen cells (Primi et al., 1977) and thymocytes (McHugh & Bonavida, 1977) in the mouse and tonsillar cells in man (Primi et al., 1977). In murine systems, antibodies have been generated agaist albumin and erythrocytes (Primi et al., 1977) as well as to DNA (Fournié et al., 1974).

A reduction in the number of B cell clones available for antibody production may be inferred from the observation of a decline in responsiveness to polyclonal activators (in vitro) with advancing age. For example, this is documented for spleen cells of BIO.5M mice after intravenous administration of endotoxin (Gronowicz & Coutinho, 1974) as well as occurring spontaneously in old NZB/NZW F_1 hybrid mice (Cohen & Ziff, 1977). This response persisted after treatment with anti-thymocyte serum plus complement, indicating that it is independent of T-cells. That the decline was in polyclonal antibody responsiveness was demonstrated in a plaque assay with hapten-conjugated sheep erythrocytes. Similar findings have been reported in old C57 BL/6 mice (Winchurch et al., 1982). Pre-treatment of young animals with endotoxin led to decreased levels of antibody synthesis which approximated to the levels found in old animals which had not been pre-treated with endotoxin. They went on to suggest that endotoxin may contribute to the genesis of immune abnormalities in aged animals and humans.

Wade and Szewczuk (1984) reported that not only does endotoxin lose its ability to suppress an ongoing immune response in vivo with advancing age but also loses its ability to enhance in vitro. They suggested that the immune system had become "down-regulated" in vivo through some prior exposure to bacterial endotoxins. Endotoxin administration in vivo has been shown to stimulate production of suppressive antiidiotypic antibody and unrelated studies have shown that such antibodies increased in aged animals (Wade & Szewczuk, 1984). This phenomenon may be a manifestation of disordered immuno-regulation, but could equally well be explained on the basis of physiological regulatory response to lymphocyte activation. Such idiotype-anti-idiotype regulation of immune responses in currently the subject of considerable interest.

The original network hypotohesis of immune regulation was advanced by Nils Jerne in 1974. The theory proposes that once the concentration of immunoglobulin molecules with a given idiotype exceeds some threshold value, B-lymphocytes which have receptors that recognise that idiotype will be stimulated to multiply and manufacture anti-idiotype antibodies. These in turn would stimulate other B-lymphocytes to make anti-anti-idiotype, and so forth. With the realisation that the anti-anti-idiotype would be structurally very similar to the original antigenic determinant, the notion of a limited rather than an infinite network became widely accepted. Furthermore, antigen-specific receptors on T-lymphocytes also possess idiotypic specificities, so they too should be included in the network. There have been many experimental demonstrations that anti-idiotype antibodies or idiotype-specific suppressor T cells can regulate immune responses and also that anti-idiotype antibody titres mirror idiotype titres in autoimmune diseases such as myaesthenia gravis and systemic lupus erythematosus. One can also conceive of the possibility that polyclonal activation could induce "anti-anti-idiotypes" de novo and that the autoantibodies which can be detected with these substances arise as a "physiological" response.

In the studies reported here, interest was directed towards "spontaneous" lymphocyte activation in aged humans. Many of the aged subjects had comparable levels of "spontaneous" lymphocyte activation to those seen in the younger controls. Nonetheless, a sizeable minority had values outside this range, some considerably so. Contrary to what was expected, the majority of these cells expressed membrane antigens typical of T-cells, although no changes in total lymphocyte populations was observed. Another interesting finding was the disparity between the expression of the T3 antigen and the T4 and T8 antigens. Two explanations are proposed. Firstly, it may be that some T-cells in the aged subjects express either T4 or T8 normally, but fail to express T3. This phenomenon has been reported for total T cell populations in peripheral blood (Hallgren et al., 1985). Alternatively, co-expression of T4 and T8 may be a feature of activated lymphocytes (Blue et al., 1985). Since the cells studied in this experiment were selected on the basis of their activation, it must remain a very strong possibility that the disparity referred to earlier merely reflects the phenomenon of lymphocyte activation. The cause of the lymphocyte activation was not proven, though evidence of endotoxin in peripheral blood was obtained in five subjects, though there was no correlation between the endotoxin level and lymphocyte activation. Considerable lymphocyte activation was observed in some subjects who displayed no evidence of endotoxaemia. This could be because the endotoxin is irrelevant, because levels which cannot be measured may still influence lymphoid function, or because endotoxin-induced lymphocyte activation occurs at a site other than the blood. The most likely candidate is the mucosal immune tissue since it is most closely apposed to the gut lumen where endotoxins are ingested and generated (colonic flora). It has been pointed out in chapter I that suppressor T-cells are generated in the gut-associated lymphoid tissue (GALT), even in normal young individuals. In this regard, it is noteworthy that the GALT shows little (if any!) functional decline with age. (Wade & Szewczuk, 1984). Alterations in the pattern of endotoxin-effects on GALT might conceivably arise if other aspects of endotoxin handling were to change with age (see chapter VII).

Plasma fibronectin was measured as an indication of reticuloendothelial function. It is known that phagocytosis of test colloids can be modulated by both immune and non-immune opsonins (Jenkin & Rowley, 1961; Saba & Di Luzio, 1969). One non-immune opsonin is fibronectin (Saba et al., 1978; Blumenstock et al., 1981). Fibronectin binds to fibrin, actin and collagen, with an exceptionally high affinity for denatured collagen (Richard & Saba, 1985) and it has been suggested that plasma fibronectin plays a major role in promoting the clearance of fibrin, collagenous debris and cytoskeletal debris following injury, thus preventing excessive localisation in highly vascular organs such as the kidneys and lung. Fibronectin behaves as an acute phase protein in rodents but this is probably not true in man.

Plasma fibronectin was determined in the study reported here and levels were found to be not normally distributed in either the elderly subjects or the younger controls. Negative skewing was a feature of the aged subjects and positive skewing was a feature of the controls. When the two groups were compared (using non-parametric tests), plasma fibronectin levels were found to be significantly higher in the older subjects. This finding is consistent with the findings of Stathakis et al. (1980). This finding is unexplained and somewhat surprising in the light of reports on modest decreases in reticuloendothelial capacity as a function of age (Brouwer & Knook, 1983).

CHAPTER VI

SURGERY AND THE IMMUNE SYSTEM

6.1 INTRODUCTION

Most of the published work in this field was stimulated by the very real problem of post-operative sepsis. Studying the immune system of humans in this setting is frought with difficulties since possible complicating factors such as the metabolic response to injury, previous nutrition, major organ failure, blood transfusion and the original disease for which the surgery was performed cannot always be controlled for. Furthermore, it is possible that some of the changes actually relate to the anaesthesia rather than the surgery.

6.1.1 Neutrophil Polymorphonuclear Leukocytes

A peripheral blood neutrophilia develops within minutes of a surgical stress and its magnitude is in proportion to the severity of the injury. It is believed that this response is induced by circulating catecholamines since it can be duplicated by the intravenous injection of adrenaline into both normal and splenectomised adult male human volunteers and probably reflects detachment of previously marginated cells (Steel et al., 1971; Gader, 1974).

Neutrophils may be adversely affected by a number of anaesthetic agents. Leukopaenia has been observed in rodents following prolonged exposure to nitrous oxide, and surgery seems to augment this effect (Parbrook, 1967). Similar findings have been reported for cyclopropane (Aldrete, 1967), pentabarbitone (Graca & Gorst, 1957), thiopentone and methohexitone (Usenik & Cronkite, 1965) and halothane (Bruce & Koepke, 1966). Bruce and Koepke (1966) also demonstrated a fall in neutrophil alkaline phosphatase activity and a fall in bone marrow proliferation _in vitro_. This is consistent with the observation of Lassen et al. (1956) that bone marrow aplasia may result from prolonged exposure to nitrous oxide in patients with tetanus.

Anaesthetic agents may also affect neutrophil function. Graham (1911), demonstrated that ether could suppress phagocytosis. In a more modern study (Matula & Paterson, 1971), halothane was shown to depress both engulfment of particles (latex beads) and metabolic activity (nitroblue tetrazolium reduction). Leck and co-workers (1974) reported that morphine and/or nitrous oxide did not affect particle uptake but did suppress nitroblue tetrazolium reduction (which is very reminiscent of the effects of glucocorticoids). It is interesting that in the two studies just cited, the anaesthetic agents consistently suppressed metabolic actvitity of neutrophils while having variable effects on particle engulfment. Hill

and co-workers (1977) reported that halothane impedes chemotaxis and random migration of neutrophils in a dose-dependent manner. Halothane depresses circulating catecholamine levels, nor-adrenaline more than adrenaline, thus giving rise to relative beta stimulation (Perry et al., 1974) and beta stimulation reduces chemotaxis whereas alpha stimulation enhances it.

Although anaesthetic agents can affect neutrophil function, their practical relevance is questionable. In the main, these effects are rapidly superceded by the direct effects of surgery (see beginning of this section). Apart from the changes in catecholamines induced by surgery, tissue damage and activation of mediator systems may also influence neutrophil function. With regard to tissue injury, a poorly defined substance named phagocytosis inhibitor (PI) has recently been described which appears to be a lipid or lipid-derivative (Huang et al., 1984). Another interesting observation worthy of note is that stimulated neutrophils from both rodents and lagomorphs can produce a protein factor which enhances lymphocyte proliferation (Goto et al., 1985).

6.1.2 The Mononuclear Phagocyte System

Within the setting of trauma, most attention has focussed upon fixed mononuclear phagocytes, particularly the hepatic Kupffer cells. Most traumatic insults are associated with depressed reticuloendothelial capacity, as assessed by the clearance of a variety of colloidal test substances from the blood (see Chapter VII for a more detailed discussion). For example, using ^{125}I labeled human serum albumin, Löfström and Schildt (1974) were able to demonstrate diminished clearance in humans exposed to cyclopropane, ether, halothane, neuroleptic analgesia and epidural anaesthesia. So uniform a response with such a wide range of agents suggests that changes in blood flow may be the most important early effect. The consequences of such an effect could well persist long after the circulation has returned to normal. It has been suggested that blood fibronectin (an opsonic glycoprotein) is a reasonable indicator of reticuloendothelial activity in the setting of surgery and trauma (Saba et al., 1978). There is also evidence that anaesthetic agents may be directly toxic to mononuclear phagocytes (Tsuda & Kahan, 1983), with cyclopropane suppressing phagocytosis (while halothane and pentabarbitone seemed to have no effect).

6.1.3 Lymphocytes

A number of changes in lymphocyte numbers and function are known to follow surgery but the precise pathogenesis of most of these changes is poorly understood. The total peripheral blood lymphocyte count falls dramatically after surgery with a nadir some 2-3 days after the peak of plasma cortisol (Hamid et al., 1984). It is felt that the two events are related, but not necessarily directly. It is known that glucocorticoids virtually "freeze" the normal circulation of lymphocytes by

arresting them in the tissues (Cox & Ford, 1982). In rats, an intravenous infusion over 15-18 hours had a much more marked effect than did a single large bolus injection. It is interesting that mild restraint in the domestic pig produces elevations in plasma cortisol levels and changes in lymphocytes similar to those seen after surgery (Westby & Kelley, 1984). This manoevre is associated with a very rapid atrophy of the thymus gland and poor DTH responses to a battery of recall antigens. Furthermore, physiological concentrations of cortisol are known to suppress the proliferative response to both PHA and ConA with minimal cytolysis. Factors other than endocrine may also influence lymphocyte recirculation. Both sodium nitroprusside-induced hypotension and femoral artery ligation produce a very rapid fall in the output of lymphocytes from popliteal lymph nodes in Rambouillet-Columbia sheep (Moore et al., 1984).

Numerous studies in man have demonstrated diminished DTH responses to a battery of recall antigens as well as to DNCB (Riddle, 1967; McLean, 1979) after surgery. The proliferative response to PHA also tends to be reduced (Riddle, 1967). Slade et al. (1975) showed that this response is much lower in the presence of autologous serum than with pooled human serum, while Bergman et al. (1981) found equivalent responses. Hamid et al. (1984) found that autologus serum actually enhanced the PHA proliferative response if tested a week after surgery. Slade and co-workers (1975) demonstrated an impaired mixed lymphocyte reaction following surgery and also that the lymphocytes from patients who had undergone surgery were much less stimulatory to allogeneic lymphocytes in pooled human serum than they were in autologous serum, indicating that a serum factor(s) could modulate the response. Apart from a transient fall in immunoglobulin and complement levels immediately after surgery (probably a dilutional effect of the infusion of crystalloids), there seems to be no consistent deficit in humoral immunity (Hamid et al., 1984).

One possible unifying thread in some of the above observations is that interleukin 2 production by unstimulated mononuclear cell cultures from humans who had undergone major surgery is impaired and if the surgical stress was great enough, this had not recovered by one week after surgery (Akiyoshi et al., 1985). There is evidence that macrophages in these patients are suppressing interleukin 2 production.

In an atttempt to clarify some of the above problems, some investigators have tried to develop animal models. Experimental gastrectomy in CBA/Ca mice is associated with diminished DTH responses but enhanced antibody production to sheep red blood cells and increased antibody production induced by pokeweed mitogen (PWM). This enhanced antibody response disappears by a week after surgery (Gryglewski et al., 1985). Laparotomy or anaesthesia alone produced no effect. They provided definite evidence that the diminished DTH responses were caused by "promiscuous" (non-specific) suppressor T cells. Other studies have confirmed such T cell mediated suppression (Munster, 1976) while yet others have

implicated B cells (Ninnemann, 1980) and macrophages (Baker et al., 1979; Wang et al., 1982).

In the face of all the above studies cited in this section stands the interesting observation that there appears to be an increase in the number of peripheral blood lymphocytes actively synthesising DNA. Ling (1968) reported an increase in the percentage of "atypical" lymphocytes in the peripheral blood after surgery. Schecter et al. (1972), Bancewicz et al. (1973) and Hamid et al. (1984) have all directly demonstrated this phenomenon by autoradiography. Blood transfusion alone and surgery alone can reproduce this effect and the effect of combining the two procedures is approximately additive. To date, no-one has attempted to characterise the nature of these cells which appear to have been activated in vivo. It is assumed that they represent a heterogeneous population since no abnormalities in the proportions of the various total lymphocyte subpopulations have been demonstrated after surgery.

6.1.4 Concluding Remarks

In conclusion, it appears that exposure to a surgical stress has well defined effects on the immune system. Functional tests of cell mediated immunity appear to demonstrate some impairment, while humoral immunity is but little affected (and may even be enhanced!). The precise mechanisms involved in the pathogenesis of these changes are poorly understood. One aspect which is of considerable interest to the theme of this monograph is the finding of an excess of "spontaneously active" lymphocytes following surgery in humans. It is already known that endotoxaemia may follow major surgery in the absence of septic complications (Thomas et al., 1982). It is also known that reticuloendothelial function (assessed by colloid clearance) is impaired, and that this impairment appears to be mirrored by changes in plasma fibronectin. The studies reported here were performed to see if these parameters might be related.

6.2 ENDOTOXAEMIA FOLLOWING MAJOR ELECTIVE SURGERY

6.2.1 Materials and Methods

Fifty patients undergoing major elective surgery were recruited over a period of six months in order to determine whether systemic endotoxaemia may occur following surgery uncomplicated by serious gram negative sepsis. Blood was drawn within 24 hours before the surgery, six hours following the surgery and on the mornings of the first, second, third and sixth post-operative days. For the purpose of this study, no patient was followed longer than six days and it is therefore not known whether some patients went on to develop any major septic complications. This endpoint was imposed because of practical considerations. Informed consent was obtained from each patient.

Limulus test.

Samples were processed and the Chromogenic Limulus test performed precisely as described in Section 2.7.3.

6.2.3 Results

Systemic endotoxaemia was identified in eight (16%) of these surgical patients. The results are summarised in Table 6.1. Samples tested on day six were also negative for endotoxin. None of the surgical patients developed major gram negative septic complications within the period of the study, though wound infections occurred in two patients. These were minor problems for the patients and only skin commensals were grown on culture.

TABLE 6.1 SUMMARY OF PATIENTS DEVELOPING ENDOTOXAEMIA IN THE FIRST THREE DAYS FOLLOWING MAJOR ELECTIVE SURGERY

Patients	Operation	Endotoxin (pg/ml)				Wound infection
		6 hr	day 1	day 2	day 3	
1	O/G	14	22	10	0	-
2	O/G	16	19	0	0	-
3	O/G	0	23	12	0	-
4	G	0	20	14	0	-
5	SB	17	15	10	0	-
6	SB	0	21	0	0	-
7	C	26	31	17	0	+
8	C	32	19	12	0	+

O = oesophagal; G = gastric; SB = small bowel; C = colon;
+ = present, - = absent

6.3 ENDOTOXAEMIA FOLLOWING SURGERY IN RHESUS MONKEYS

6.3.1 Introduction

It was decided to study post-operative endotoxaemia in rhesus monkeys rather than in rodents, since rhesus monkeys may be more representative of the situation in man. Furthermore, the surgical procedures were performed under similar circumstances to those employed in human surgery.

6.3.2 Materials and Methods

The results reported here derive from two groups of young adult rhesus monkeys which had undergone experimental organ transplantation in the Primate Centre (TNO). The first group comprised seven animals which had undergone experimental liver transplantation without histocompatibility testing. Blood from these seven animals was obtained pre-operatively and the plasma used to determine the enhancement and inhibition characteristics of heparinised rhesus monkey plasma in the chromogenic Limulus Test (Chapter II). Four animals (which had been hyper-immunised against donor antigens prior to liver transplantation) died of hyper-acute rejection and no samples were available for analysis. The remaining three animals received cyclosporin-A (10 mg/kg) and prednisolone (1 mg/kg) daily for duration of the study.

The second group of animals comprised 5 kidney donors. Apart from their unilateral nephrectomy, this group of animals received no other treatment. Animals were bled by femoral venepuncture at the completion of surgery and thereafter at daily intervals for 2-3 days and then at approximately 3 day intervals. The frequency of sampling is made clear in the results section. Heparin was used as anticoagulant (see Chapter II). Blood was processed immediately for the chromogenic Limulus test. On the basis of the enhancement/inhibition characteristics shown in Chapter II, a 1 in 20 dilution of plasma combined with heating at 75°C for ten minutes was chosen for the assays. Standards were prepared in the same dilution of pooled pre-operative monkey plasma.

6.3.3 Results

All three of the monkeys undergoing experimental liver transplantation developed endotoxaemia (Table 6.2). Two animals died from operative complications without any evidence of rejection. Only one of these animals was endotoxaemic at the time of death.

Of the renal transplant donors, 4 animals developed post-operative endotoxaemia and the results are summarised in Table 6.2. Results for later time points (not shown in the table) were all negative.

6.4. LYMPHOCYTE ACTIVATION AND ENDOTOXAEMIA IN HUMAN PATIENTS UNDERGOING MODERATE/MAJOR ELECTIVE SURGERY

6.4.1 Materials and Methods

Fifteen patients undergoing moderate/major elective surgery were recruited into the study. Nine subjects underwent oesophageal and/or gastric surgery, three underwent biliary tract surgery and three underwent small bowel surgery. No patient was jaundiced or had evidence of infection at the time of the study. Blood was drawn before going to theatre, within six hours of the operation and on the

mornings of days 1, 2, 3 and 6. Informed consent was obtained from each patient.

Besides any routine investigations, the following investigations were performed: spontaneous incorporation of tritiated thymidine; chromogenic Limulus test (daily from days 0-6); fibronectin determination (days 0, 1, 3 and 6); peripheral blood T lymphocyte characterisation.

TABLE 6.2 SUMMARY OF ENDOTOXIN MEASUREMENTS (EU/ml) IN YOUNG ADULT RHESUS MONKEYS IN RELATION TO SURGERY

Operation	Animal	Endotoxin concentration (EU/ml) on days:							
		0	1	2	3	4	5	6	7
Liver transplant	1	0	0.425	0.3	0.33	0	0	0	0
	2	0	1.24	-	-	-	-	-	-
	3	0	0.325	0.55	0.74	0	0	0	0
Nephrectomy	1	0	0.295	0	0	0	-	-	-
	2	0	1.55	1.45	0.49	0	0	0	0
	3	0	1.446	0.54	0	0	0	0	0
	4	0	0.31	0	0	-	-	-	-
	5	0	0	0	0	-	-	-	-

6.4.2 Results

6.4.2.1 Spontaneous incorporation of tritiated thymidine

This study confirms the findings of Hamid et al (1984) that there is a significant excess of "spontaneously active" lymphocytes some 6 days after surgery, and that scintillation counts tended to be higher in subjects who have also undergone blood transfusion. The results are summarised in Table 6.3, though the results of surgery alone and surgery with blood transfusion are not given separately since the number of subjects is small.

TABLE 6.3 SUMMARY OF RESULTS OF FIBRONECTIN ESTIMATION AND INCORPORTATION OF TRITIATED THYMIDINE FOR 15 PATIENTS UNDERGOING MODERATE/ MAJOR ELECTIVE SURGERY

	Day 0	1	3	6
Mean fibronectin (expressed as % of basal value	100%*	61% (°$p < 0.01$)	76% (°$p < 0.01$)	112% (°$p < 0.01$)
Mean thymidine incorporation (counts/minute)	420 (±128)	–	-	1570 (±268) (°$p < 0.01$)

Results expressed as means. ± related to standard error.
* The actual mean value for the day 0 plasma fibronectin level was 32.71 mg/dl.
° Statistical analysis by Kruskall-Wallis one way analysis of variance.

6.4.2.2 Fibronectin determinations

The results of the fibronectin determinations for the whole group are given in Table 6.3. There is a significant fall from the pre-operative (basal) level by day 3 ($p < 0.01$) with a significant rebound above the pre-operative level by day 6 ($p < 0.01$). Every patient demonstrated this same pattern of response. Data for the four endotoxin-positive subjects is given separately in table 6.4. The values given for each time point do not significantly differ from the values seen for the whole group or from those seen in the eleven endotoxin-negative subjects (data not shown).

6.4.2.3 Endotoxin determinations

Endotoxin was detected in four of the fifteen subjects (26%). Endotoxaemia was detected within six hours of surgery in all four cases and was undetectable in all four cases by the third post-operative day. One subject was free from endotoxaemia within 24 hours and another within 48 hours. Three of these patients had undergone Nissen fundoplication procedures. All three were obese and the surgery took longer than usual. The fourth subject was a sixty four year old male who underwent elective cholecystectomy and whose post-operative course was punctuated by a pneumococcal pneumonia but with negative blood cultures. The other three subjects were discharged on the seventh post-operative day and were free from septic complications. Data on fibronectin determinations and lymphocyte activation in these four patients are given in Table 6.4.

TABLE 6.4 SUMMARY OF RESULTS OF FIBRONECTIN ESTIMATION AND SPONTANEOUS THYMIDINE INCORPORATION IN FOUR PATIENTS WITH DETECTABLE POST-OPERATIVE ENDOTOXAEMIA

	Day					
	0	6 hrs	1	2	3	6
Mean fibronectin (expressed as % of basal value	100%*	-	57%	-	75.75%	112%
Mean thymidine incorporation (cpm)	460 (± 131)	-	-	-	-	1822 (± 273)
Mean Endotoxin (pg/ml)	0	20.0	11.5	5.75	-	-

* The actual mean value for the day 0 plasma fibronectin level was 31.92 mg/dl.

6.4.2.4 T Lymphocytes

The results of the T lymphocyte characterisation studies on peripheral blood mononuclear suspensions are given in Table 6.5. No significant change (Mann-Whitney U test) occurred between the pre-operative values and those seen on day 6. This suggests that the "spontaneously active" lymphocytes are likely to represent a heterogeneous population.

TABLE 6.5 MEAN PERCENTAGE OF CELLS EXPRESSING T CELL SURFACE ANTIGENS IN MONONUCLEAR CELL SUSPENSIONS FROM THE PERIPHERAL BLOOD OF 15 SURGICAL PATIENTS BOTH BEFORE SURGERY AND ON THE SIXTH POST-OPERATIVE DAY

Surface antigen	Day 0	Day 6
T3	74.5 (± 4.26)	76.2 (± 5.31)
T4	51.2 (± 3.92)	48.9 (± 4.30)
T8	28.1 (± 3.24)	27.6 (± 3.12)

Figures in brackets represent standard deviation.

6.5 DISCUSSION

It is clear from the results reported above that endotoxaemia can and does follow moderate-major surgery in both humans and rhesus monkeys. Endotoxaemia was seen in 16-26% of the human patients, in 80% of the rhesus monkey kidney donors and in 100% of the rhesus monkey liver transplant recipients. The actual levels of endotoxin were entirely comparable between the humans and the monkeys. The questions that spring immediately to mind are:

1. Why should endotoxaemia develop post-operatively?
2. Why should the frequency be so different between humans and rhesus monkeys?

Endotoxaemia has been demonstrated following thermal injury in mice (Markley et al., 1971) and translocation of bacteria accross the gut wall has been reported following thermal injury in rats (Maejima et al., 1984). Thermal injury is associated with complex changes in the distribution and control of blood flow. Similar changes follow surgery and appear to be aimed at maintaining perfusion of essential organs such as the brain, lung, liver and kidneys. Flow to less critical organs and tissues such as the gut and adipose tissue is reduced. Anaesthetic agents may themselves induce haemodynamic changes and these have been summarised by Vickers et al. (1978):

1. Myocardial depression - by a direct effect on the myocardium
2. Increased myocardial irritability - resulting in dysrhythmias.
3. Tachycardia (sinus)
4. Decreased cardiac output with a redistribution of peripheral blood flow.
5. Hypotension in euvolaemic subjects during moderate anaesthesia and in hypovolaemic subjects with mild anaesthesia.

Hypoperfusion of the intestine might be responsible for enhanced endotoxin absorption into the portal blood, or even lymph. In a recent and as yet unreported study (K. Braim, personal communication), systemic endotoxaemia was shown to develop in rats whose distal colon had been rendered avascular following ligation of the relevant arteries and veins. It is suggested that the endotoxin leaked through the gut wall into the peritoneal cavity and eventually reached the systemic circulation via lymph. It is also possible that changes in liver blood flow might impede endotoxin removal (see Chapter VII). During mechanical trauma, total liver blood flow is well maintained, but becomes relatively more dependent upon the hepatic arterial contribution. It should be recalled from Chapter I that hepatic arterial blood may enter some way down the sinusoid, thus by-passing a proportion of the Kupffer cells. Furthermore, shunts are known to exist between hepatic arterioles and hepatic venules. These would have the effect of diverting some blood from the sinusoids.

Anaesthetic agents themselves may suppress reticuloendothelial system (RES) activity (see Chapter VI, section 6.1.2). Since this effect is seen with morphine

and epidural anaesthesia as well as with general anaesthetics, it is suggested that changes in blood flow may be more important in the generation of RES depression than is direct toxicity.

Other factors may also change RES activity. Reticuloendothelial blockade is a phenomenon which can be induced by the administration of large doses of colloidal materials. Indeed, some of the test colloids used for the assessment (even clinical assessment) of RES activity may have this property. Early studies (Benecerraf et al., 1975) suggested that RES blockade resulted from some change in the phagocytic cells. Other studies (Saba & Di Luzio, 1969) suggest that post-traumatic RES blockade arises from the depletion of the opsonic glycoprotein, fibronectin. Blumenstock et al. (1979) reported that the observed RES activity correlates directly with the observed concentration of fibronectin. Scovill et al. (1977) report that the concentration of fibronectin drops following surgical trauma, suggesting an impairment in RES activity. The study reported here confirms that the concentration of plasma fibronectin falls very rapidly after surgery. One might speculate that this fall is due to its binding to tissue debris and the complexes are taken up by Kupffer (and other RES) cells. The time course of RES depression and a fall in fibronectin concentration seem not to be directly associated. Allowing significant trauma, RES activity drops very rapidly but recovers (often to supra-normal levels) within 24-48 hours (Altura, 1980). It is clear from this study that by 48 hours, plasma fibronectin is still depressed (but is increasing). Plasma fibronectin levels are elevated on the sixth day after surgery. These observations suggest to the author that RES activity and fibronectin concentration are not directly related in the way that has been previously proposed.

Endotoxin may influence fibronectin concentration under certain circumstances. Richards and Saba (1985) injected adult male Sprague-Dawley rats intravenously with 1 mg of endotoxin on days 1, 3 and 5. This resulted in a 100% increase in fibronectin concentration within 24 hours with a return to normal by day 5. RES clearance capacity (assessed by test colloids) was enhanced on both day 3 and day 5, thus directly demonstrating that fibronectin levels do not reflect RES activity following endotoxin administration.

The second question posed at the beginning of the discussion was "why should the frequency of endotoxaemia be so different between humans and rhesus monkeys?". Species differences apart, this phenomenon could be due to differences in the type of surgery or to differences in the circumstances in which the surgery was performed. With reference to the type of surgery, it is of interest that all three liver transplant recipients developed endotoxaemia. At present, there is great interest (but little information) on the fate of the transplanted Kupffer cells. It has been suggested (but not proven) that they are soon replaced by cells of host origin. One could further speculate that this replacement phase may lead to a temporary impairment of hepatic reticuloendothelial functions. In connection with the circumstances of the surgery, one explanation might be that human subjects

undergoing surgery are subjected to more rigorous haemodynamic monitoring and intervention than is the case for the monkeys. This would tend to limit the effects of the haemodynamic changes in the human subjects.

Before moving on from the subject of the mechanisms which might be involved in the production of post-operative endotoxaemia, it is important to clearly state the author's view. This is that haemodynamic changes following surgical trauma enhance endotoxin absorption and reduce its elimination (by reducing RES activity). RES blockade may follow the uptake of large amounts of tissue debris (complexed to fibronectin). Direct anaesthetic toxicity may also be involved. These views are summarised in Figure 6.2.

The final part of this discussion relates to the lymphocyte activation and its possible relationship with endotoxaemia. This study confirms the findings of others (Ling, 1968; Schecter et al., 1972; Bancewicz et al., 1973; Hamid et al., 1984) that there is a post-operative increase in the number of "spontaneously active" lymphocytes following moderate-major surgery. Blood transfusion also produces the same phenomenon, probably because of histo-incompatibility of transfused white blood cells. No attempt was made in the present study to separate the effects of blood transfusion and surgery alone (because of the small number of patients).

It is suggested that the circulating endotoxin may contribute to the lymphocyte activation. Chapter I summarises the immunological properties of endotoxins and thereby makes clear the potential link between endotoxin and lymphocyte activation. Since all subjects gave evidence of lymphocyte activation, why didn't all subject have circulating endotoxins? The most obvious complicating factor is that 12 of the 15 subjects had received a transfusion of blood (including all four endotoxin-positive subjects).

Furthermore, it may be that some of the other subjects may have had circulating endotoxin at a concentration below the sensitivity of the endotoxin assay. It has been made clear in the introduction that concentrations of endotoxin as low as 5 pg/ml can influence the mixed lymphocyte reaction. Lastly, if endotoxin absorption from the gut is increased, and the capacity for its uptake impaired, one can envisage the possibility of lymphocyte activation within the liver itself.

Attempts were made to characterise the nature of the "spontaneously active" lymphocytes but suitable preparations were available from only six of the subjects (data not shown). In these subjects, the majority of the "spontaneously active" lymphocytes were T lymphocytes which bore the surface antigens associated with cytotoxic/suppressor cells. If these data are representative, it is unlikely (though not impossible) that they were generated directly by endotoxin but may represent a suppressor response to B cell activation. It should be noted that endotoxin is certainly implicated in the generation of suppressor T cells in vivo (Chapter I, section 1.2.9).

The nature of these "spontaneously active" cells is clearly of great interest. The technique reported in this monograph it too cumbersome to apply to large

numbers of patients and it is only possible to examine a very small number of cells. Perhaps a potentially more productive approach would be to use a fluorescence-activated cell sorter to prepare populations of cells and then to perform autoradiography on these populations.

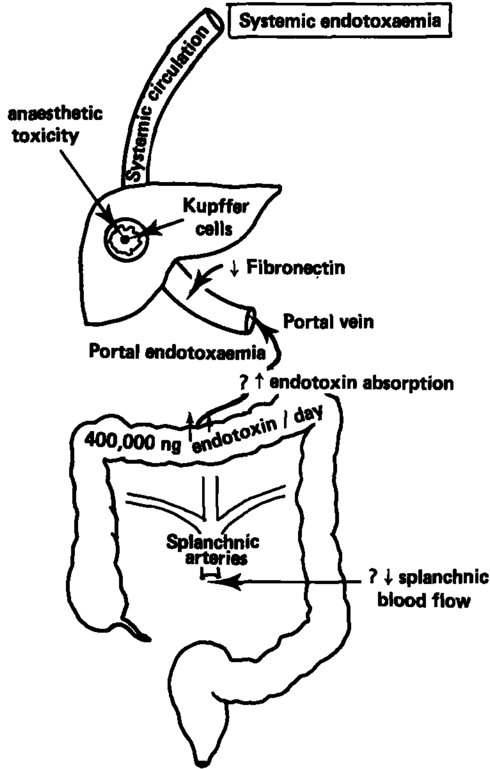


Fig. 6.2 Diagram illustrating the events which may contribute to post-operative endotoxaemia.

CHAPTER VII

ENDOTOXIN HANDLING AND AGE-RELATED CHANGES

7.1 INTRODUCTION

In this penultimate chapter, the issue of endotoxin handling and how it might change as a function of age, will be addressed. This topic was first raised in Chapter I. Sufficient detail was given insofar as it was relevant to Chapters IV-VI. It is now appropriate to tackle the issue in much more depth, since this will allow the reader to view the experiments to be presented later in the correct context. However, only those issues which arise as a direct consequence of these experiments will be discussed in the final section of this chapter (Section 7.4)

7.1.1 The Fate of Injected Endotoxins

Two basic approaches may be applied to follow the fate of injected endotoxins:

1. Chemical or biosynthetic labeling of endotoxin, usually with a radioactive isotope, and then following the disappearance of the label from plasma and its appearance in tissues.
2. Injection of unlabeled endotoxin with its subsequent detection by chemical or immunological means or directly by electron microscopy.

Most reported studies employ the first of these approaches and in interpreting them, the possibility of re-utilisation of the label must be considered. All studies of this type (e.g. Mathison & Ulevitch, 1979a; Mathison et al., 1980) demonstrate a biphasic disappearance curve following intravenous bolus injection of labeled endotoxin, regardless of the species of animal employed for the experiment (Fig.7.1).

The initial phase of rapid decline has a half life of about 30 minutes and is followed by a much slower phase with a half life of several hours. Accurate estimates of this later phase could not be made in any of these studies since experiments were terminated before the completion of one half life.

The shape of the curve is strongly reminiscent of the curves obtained for drugs whose elimination follows first order kinetics. For such drugs, the initial rapid phase represents distribution and the slower phase represents elimination. Elimination is characterised by estimating the half life ($t\frac{1}{2}$). If this elimination curve is extrapolated to the ordinate, a value for concentration is obtained which represents the concentration that would have been achieved if distribution were instantaneous. By dividing the injected dose by this value, the apparent volume of distribution (Vd) can be calculated. Both the $t\frac{1}{2}$ and the Vd govern the clearance

(Cl) of the drug. These three parameters are related in the equation:

$$t_{\frac{1}{2}} = \frac{0.693 \times Vd}{Cl}$$

(units of time)

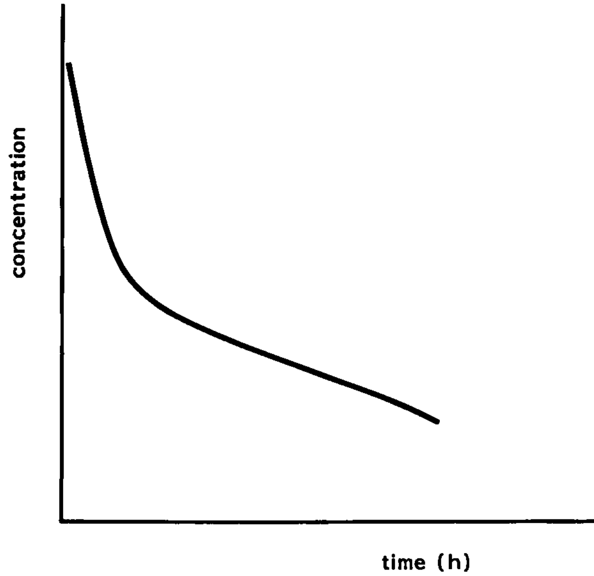


Fig. 7.1 Diagrammatic representation of an endotoxin blood disappearance curve. (for explanation, see text).

Clearance can also be calculated under steady state conditions when a drug is infused at a constant rate. The concentration of drug at the steady state (Css) depends only on the infusion rate (Rinf) and the clearance, as defined by the equation:

$$Css = \frac{Rinf}{Cl}$$

(amount per unit volume)

If one attempts to calculate values for endotoxin clearance using the data published in the literature, the values obtained are very inconsistent and compare very unfavorably with the value obtained under steady state conditions (0.906 ml/min) using data from the only steady state experiment available (Fish & Spitzer, 1984). This suggests that the assumption that endotoxin clearance can be considered as a process which follows first order kinetics, is invalid.

Evidence that the early phase of the plasma elimination curve does not represent distribution comes from the fact that considerable quantities of the injected dose of endotoxin localise very rapidly in the liver (section 7.1.3). Clearly, an alternative explanation must be sought to explain the shape of the elimination curve. Four possible explanations for the biphasic nature of the elimination curve (which are not mutually exclusive) are immediately apparent:

1. Following injection, the endotoxin preparation is changed in some way, and this change modifies clearance.
2. Following injection, endotoxin itself can modify its own clearance, either by a direct effect on the cells responsible for its clearance or by inducing changes in local blood flow or in plasma composition.
3. The endotoxin preparations may be very heterogeneous with several physical (or even chemical) forms being present simultaneously. It can be envisaged that clearance mechanisms may differ for different forms.
4. The label may be re-utilised.

Direct evidence exists only for the first of these proposals (Section 7.1.2). Nonetheless, there is indirect evidence which is in support of the other three. In experiments in which endotoxin is administered via the portal vein, very definite changes in portal wedge pressure, portal vein plasma flow as well as in systemic blood pressure, have been demonstrated (see Section 7.1.5). These observations relate to the second possibility listed above. Endotoxin can also modulate the clearance of a number of RE test substances and the induction of fibronectin (an acute phase protein in the rat, but not in man!) may be important in this regard (Richards & Saba, 1985).

Following equal doses (on a weight basis) of endotoxin preparations from smooth and rough strains of bacteria, far more of the endotoxin from the rough strain disappears from the plasma during the early, rapid phase of the plasma disappearance curve. Endotoxin preparations from rough strains tends to exist in larger aggregates than that from smooth strains (Mathison & Ulevitch, 1979). Beer et al. (1965) separated a preparation of E.coli 0:113 endotoxin (Boivin preparation) on a sucrose density gradient and showed that this preparation was very heterogeneous in terms of density, and that endotoxin prepared from different parts of the gradient exhibited very different potencies. The observations which have just been discussed relate to the third of the possibilities listed above. Furthermore, one might speculate that the denser (larger) particles may be more readily taken up by phagocytic cells.

Evidence for the last of the proposals (re-utilisation of label) comes from some double biosynthetic labeling experiments in which a smooth form endotoxin was labeled with both ^{3}H and ^{14}C (Freudenberg et al., 1984). The ^{3}H was incorporated exclusively in the fatty acids while the ^{14}C preferentially labeled polysaccharide (see Section 7.1.4). The result demonstrated that ^{3}H was excreted preferentially from the organism over a fourteen day period. It seems unlikely that the ^{14}C (which represents mainly polysaccharide) was stored in cells for such a prolonged period and one must seriously consider that the label may have been re-utilised. Indeed, it appears from this very experiment that ^{14}C was not stored at the initial site of uptake.

7.1.2 Changes in Endotoxins after Injection

A change in endotoxin following intravenous injection has been reported by several authors. The change is due to an interaction with plasma lipoproteins and results in a change in the buoyant density of the endotoxin preparation (Ulevitch & Johnson, 1978). Using an ^{125}I-labeled phenol-water extract of E.coli O111:B4 (which is fully active), they demonstrated that the change is very rapid and within 30 minutes of injection, over 80% of the endotoxin remaining in plasma had undergone a change in buoyant density to a low density form (Mathison & Ulevitch, 1979). If this low density form is removed and concentrated (by dialysis against physiological saline) and then injected into another animal, the initial rapid phase of the disappearance curve (see Fig. 7.1) is lost. Nonetheless, the disappearance curve remains biphasic; now with an initial half life of about 6 hours followed by a slower phase of elimination with a half life of about 15 hours. This clearly shows that the change which occurs in blood that results in a change in buoyant density can also result in a change in the kinetics of endotoxin removal, though it was not demonstrated that the endotoxin remained bound to the lipoprotein during the dialysis procedure.

Subsequent studies (see Maier et al., 1981; Freudenberg et al., 1984) have increased our understanding of this interaction. First of all, there are some non-lipid plasma components which facilitate this interaction, possibly by disaggregating the endotoxin. It is also known that this binding is almost exclusively to high density lipoprotein (HDL). Endotoxin-HDL interaction reduces the ability of mouse bone marrow derived macrophages to take up endotoxin but enhances endotoxin localisation in the adrenal cortex (where the endotoxin is seen in the cytoplasm of cortical epithelial cells (Freudenberg et al., 1984). The endotoxin-HDL interaction does not abort endotoxin toxicity in mouse lethality assays, though it does reduce the capacity to induce fever, neutropaenia and complement activation (Mathison & Ulevitch (1981). This may mean that the manifestations of endotoxin-toxicity are not directly related to the ability of an organism to clear it.

Under appropriate conditions, binding to proteins other than HDL can be demonstrated, both in the rabbit (Tobias & Ulevitch, 1983) and in man (Tobias et al., 1985). Acute phase serum was induced with etiocholanolone. An endotoxin preparation from Salmonella minnesota Re 595 formed a complex with a density different from what is observed in normal serum and the rate of formation of this complex is at least ten times slower than the HDL-endotoxin interaction. The identity of this acute phase protein(s) is unknown but appears not to be C-reactive protein or serum amyloid A.

Active endotoxin preparations (but not radio-detoxified endotoxins) result in hyperlipidaemia in a number of mammalian species (Gaal et al., 1984). This is characterised by marked elevation of triglyceride-rich VLDL (very low density lipoprotein). The elevation in HDL was only modest (30% over control) and some authors (Sakaguchi, 1982) record a fall in HDL. Over the same time course (18-24 hours), lipid droplets were deposited in liver parenchymal cells. Changes in HDL might be expected to modify endotoxin clearance.

7.1.3 Distribution and Localisation of Injected Endotoxins

Numerous studies have used isotopically-labeled endotoxin preparations to establish the organ distribution and cellular localisation of injected endotoxins (reviewed in Mathison & Ulevitch, 1979). In their own study, ^{131}I-labelled E.coli O111:B4 endotoxin (phenol-water extract) was injected into male NZB rabbits. Some 45% of the injected dose could no longer be detected in the peripheral blood five minutes following injection and by 180 minutes, 66% had disappeared. At both time points, the bulk of the "tissue-bound" endotoxin was located in the liver, though the concentration in the adrenal was higher than in the liver. Broadly similar results were also found for ^{131}I-labeled Salmonella minnesota Re 595 endotoxin (aqueous phenol-chloroform- petroleum ether extract) which is a preparation lacking the long polysaccharide O antigen. Autoradiography of liver sections localised the radioactivity to the sinusoidal lining cells. Electronmicroscopic autoradiography showed that the radioactivity was concentrated within phagocytic vesicles of Kupffer cells but with significant amounts being associated with mitochondria and cytoplasm. It is not known whether the radioactivity found in the cytoplasm or associated with mitochondria actually represents the presence of endotoxin or whether the label had already been removed. It is certainly possible that the endotoxin had been degraded in the phagocytic vesicles and only the label was found elsewhere.

Studies using immunocytochemical methods to identify endotoxin have allowed some estimation of the likely time course of events within the liver (Freudenberg et al., 1982). The endotoxin preparations used in this study were Salmonella abortus equi (phenol-water extract) and Salmonella minnesota Re 595 (phenol-chloroform-petroleum ether extract). The preparations were detected by affinity purified rabbit antibodies directed against the O antigen and the Re antigen respectively.

These antibodies were detected by peroxidase labeled pig anti-rabbit IgG. Sublethal doses of endotoxin were injected intravenously into specific pathogen free, female, AS2 rats (3 months of age) These rats were known to possess no natural antibodies against the O or Re antigens in question, which is critical for the validity of the results. Animals were killed at various intervals over nine days and perfusion fixed through the left ventricle. For the smooth endotoxin preparation (Salmonella abortus equi), only sporadic peroxidase staining could be observed at two hours post-injection. By seven hours there was heavy staining of sinusoidal lining cells which had often ingested other cells (polymorphonuclear leucocytes and erythrocytes). By day three, the staining-intensity of sinusoidal lining cells was declining and endotoxin could now also be detected within parenchymal cells. By day nine, almost all peroxidase staining had disappeared. In contrast to this, the endotoxin from the rough strain (Re 595) showed strong peroxidase activity in both sinusoidal lining cells and parenchymal cells within 30 minutes of injection. This pattern persisted for 48 hours, whereafter it declined so that virtually no staining could be seen by five days. Almost identical findings were observed by the same authors using double labeling of endotoxin with ^{3}H and ^{14}C (Freudenberg et al., 1984). The model which these authors proposed is summarised in Figures 7.2a and 7.2b. The concept that cells of the mononuclear phagocyte system play an important role in endotoxin elimination is not new and may be traced back to the observation of Beeson in 1947 (reviewed by Di Luzio et al., 1980) that rabbits previously rendered resistant to the febrile and lethal effects of endotoxin (by an appropriately timed exposure to endotoxin itself) could be resensitised by administering a large dose of a colloidal material which rendered the reticuloendothelial system hypofunctional.

It has been reported that some smooth endotoxin preparations can localise very rapidly in hepatic parenchymal cells (Zlydaşzuk & Moon, 1976). Mice were injected with ^{51}Cr-labeled Salmonella typhimurium LPS and liver cells were isolated one hour later by enzymic digestion. Endotoxin present in parenchymal cells was measured by lysing them with pronase and counting radioactivity in the supernatant. One problem with this study is that the initial cell isolation was performed at 37°C for 30-45 minutes. Transfer of endotoxin (or radioactive label) from Kupffer cells to parenchymal cells could therefore have occurred. Another study by Maitra et al. (1981) also suggests that large amounts of injected endotoxin localise very rapidly in hepatocytes. In their study, the endotoxin was detected directly by measuring β-hydroxymyristic acid (from the lipid A moiety of endotoxin) by a gas chromatography/mass spectroscopy method. Although large quantities of β-hydroxymyristic acid were found in the purified hepatocytes, it must be pointed out that the cell isolation procedures were also performed at 37°C and transfer of endotoxin from Kupffer cells to hepatocytes could not be ruled out. Nonetheless, binding of endotoxin to isolated hepatocytes has been demonstrated (Ramadori et al., 1979).

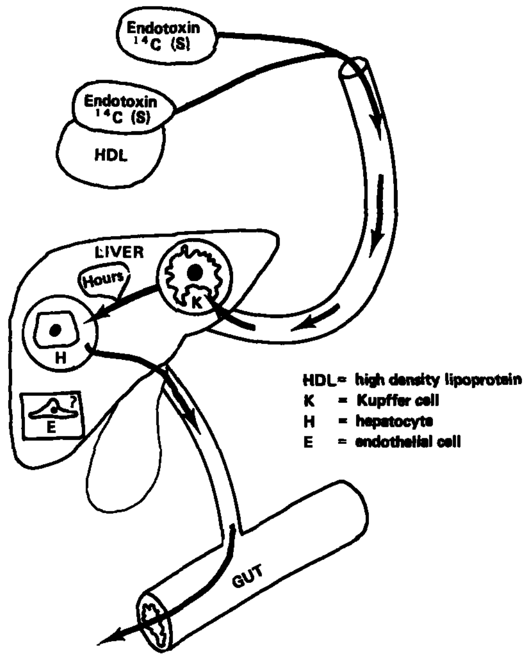


Fig. 7.2a Scheme for the hepatic localisation of a smooth-form endotoxin in the rat (after Freudenberg et al., 1984).

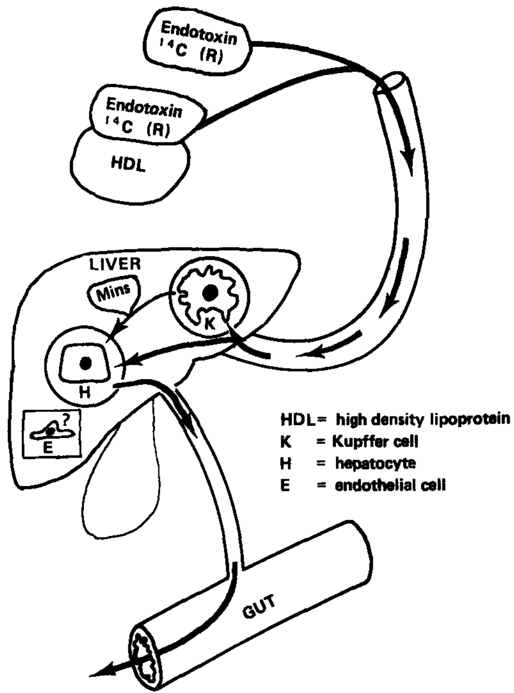


Fig. 7.2b Scheme for the hepatic localisation of a rough-form endotoxin in the rat (after Freudenberg et al., 1984)

7.1.4 Removal of injected Polysaccharide moiety and Whole Bacteria from Blood

Injection of ^{125}I-labeled polysaccharide of Salmonella type into NZW rabbits follows the same general pattern as that for an extracted endotoxin preparation (Fig. 7.1) except that the time course is different (Isibasi et al., 1983). The initial rapid phase has a $t\frac{1}{2}$ of approximately 6 minutes and the slower phase has a $t\frac{1}{2}$ of approximately 55 minutes. The tissue distribution and localisation of the labeled polysaccharide was very similar to that described for extracted endotoxin preparations (Section 7.1.3) except that the amount appearing in the urine was greater. These results led the authors to conclude that the polysaccharide moiety of endotoxin may be an important determinant of endotoxin clearance. Other studies (Perry & Ofek, 1984) suggested that certain sugars and glycoproteins play an important role in the removal of whole live bacteria following intravenous injection. Clearance was inhibited by co-injection of methyl-α-D-mannoside, methyl- α-D-glucoside and methyl-α-D-fucoside but not by methyl-α-D-galactoside or L-rhamnose. Likewise, clearance was inhibited by asialofetuin or ovalbumin but not by fetuin. Homogenates of mouse liver co-aggregated with non-fimbriated E.coli and this aggregation could be blocked by 100 mM solutions of methyl-α-D-mannoside, methyl-α-D-glucoside and 1 mg of extracted lipopolysaccharide but not by L-rhamnose. They interpreted these results by suggesting that the mannose-N-acetylglucosamine hepatic lectin recognises specific sugars on the surface of E.coli and may be significantly involved in the non-immune clearance of this organism.

7.1.5 Endotoxin Detoxification

Studies on this topic were facilitated by studies examining endotoxin sensitivity and resistance. Wide variations exist among commonly used species of laboratory animals in their innate sensitivity to endotoxins. McCusky et al. (1982) suggests that this innate sensitivty or resistance relates to the relative numbers of Kupffer cells present in the liver. Unfortunately the laboratory rat is extraordinarely resistant to endotoxins, when compared to some other mammalian species (Table 7.1)

However, a number of chemicals can alter innate endotoxin sensitivity. For example, appropriate doses of lead acetate have been shown to increase the sensitivity of the rat to endotoxin-induced lethality by a factor of greater than 2×10^3 (Trejo & Di Luzio, 1979). This means that the lead-sensitised rat can be used as a bioassay for endotoxin which is sensitive over the range of 0.3 - 3.0 μg/rat. Likewise, actinomycin-D has been shown to increase the sensitivity of mice by a factor of more than 10^5 and permit measurements in the range 0.01 - 1 ng/mouse (Pieroni et al., 1970). Filkins (1970) used the lead acetate-sensitised rat model to assess the ability of various organ homogenates to detoxify endotoxins. Liver and spleen homogenates had a large capacity to detoxify endotoxins, the lung had only a modest capacity and the brain and kidney were equally ineffective.

TABLE 7.1 RELATIVE SENSITIVTY TO ENDOTOXIN-INDUCED LETHALITY BETWEEN SOME SPECIES OF EXPERIMENTAL ANIMAL (after Filkins, 1970)

Animal species	Relative LD_{75} * (arbitrary units)
Cat	1
Rabbit	2.5
Dog	8
Guinea pig	20
Rat	300

* LD_{75} refers to the dose that leads to death of 75% of the animals to which it was administered.

Further studies by the same author (Filkins, 1971) compared the capacity of cells of the granulocyte lineage with that of a number of representatives of the mononuclear phagocyte system, to detoxcify endotoxins. Sonicates of granulocytes displayed little capacity for detoxification while sonicates of both peritoneal exudate macrophages and Kupffer cells displayed a marked detoxification potential.

Rippe et al. (1974) injected mice with 250 µg of endotoxin and the animals were then sacrificed at intervals. Liver suspensions were then injected intraperitoneally into other groups of mice and lethality assessed over 24 hours. Mice injected with liver removed 15 minutes after being injected with endotoxin displayed 88% mortality. This fell to 36% when a 6 hour sample was injected and was almost nil with a 24 hour sample. This makes one wonder whether the endotoxin which persists in the liver for days or more following injection (Section 7.3) is actually active.

Kleine et al. (1985) adopted a somewhat different approach in order to assess detoxification in vivo. Using double biosynthetic labeling of Salmonella abortus equi with ^{3}H an ^{14}C, all the ^{3}H was located in the fatty acids while ^{14}C preferentially labeled the polysaccharide. Phenol-water extracts of this endotoxin were injected into AS2 rats intravenously under anaesthesia. Eighteen percent of the injected ^{3}H and 32.1% of the injected ^{14}C were recovered in the urine and faeces over 14 days. These relative quantities of label as well as the relative quantities found in the liver at 14 days were quite different from the relative amounts of label in the original endotoxin and it was inferred that fatty acids were selectively removed.

Similar results were obtained by Munford and Hall (1985) who demonstrated deacylation of Lipid A following uptake of endotoxin by elicited murine peritoneal macrophages. Furthermore, the capacity to take-up and deacylate endotoxin did not differ between endotoxin-sensitive (C3H/HeN) and endotoxin-resistant (C3H/HeJ) mouse strains. Since most of the toxic properties of endotoxins are concentrated in the lipid moiety, this may be taken as indirect evidence of detoxification. This also suggests that metabolism of lipid A is not a major determinant of endotoxin sensitivity.

Maitra et al. (1981) were able to show (by measuring β-hydroxymyristic acid) that more than half of the injected dose could be found in the liver within three hours of injection into mice. Since the distribution of this recovered endotoxin in Folch extraction was similar to what is observed when intact endotoxin was analysed separately, it was presumed that the endotoxin was not significantly altered. β-hydroxymyristic acid could be detected in the bile within 30 minutes of injection and around 7% of the injected dose was excreted over 48 hours. This excreted β-hydroxymyristic acid was found to be associated with polar and non-polar metabolites of endotoxin.

7.1.6 Fate of Endotoxin Presented via the Portal Vein

Wolter et al. (1978) attempted to quantify removal of endotoxin by the liver both when infused into the hepatic artery and into the portal vein. Minipigs (16-22 Kg body weight) were anaesthetised under N_2O/O_2 and infused with E.coli O26: B4 endotoxin (Difco) at a dose of 0.16 mg/Kg over 28 minutes, either via the hepatic artery or portal vein. Hepatic venous blood was collected through an indwelling cannula and later subjected to a Limulus gellation test. With hepatic artery infusion, the test became positive within four minutes (11.4 μg of endotoxin/Kg body weight was extracted before spillover occurred). With portal vein infusion, the test remained negative until 12 minutes (corresponding to 57 μg/Kg body weight extracted before spillover). Transit times through the liver following hepatic artery and portal vein injection were assessed by injecting indocyanine green and gave values of 3.6 seconds and 8.3 seconds, respectively. This study shows that the dynamics of liver blood flow may well be of some importance in the removal of endotoxins. There is reasonable agreement that the extraction efficiency of Kupffer cells is inversely related to liver blood flow (see Wolter et al., 1978). In a normal liver of most species tested, some 6% of total blood flow bypasses the sinusoids and shunts have been demonstrated between the portal vein and the hepatic vein and between the hepatic artery and the hepatic vein. Furthermore, not all the hepatic arterial blood joins the sinusoids in the peri-portal region but may join at other arterial-sinusoidal junctions so that a proportion of Kupffer cells may be excluded from exposure to hepatic arterial blood. Furthermore, differences occur both in the number and in some functional aspects between Kupffer cells in the peri-portal region and those in the peri-central region of liver lobules

(Sleyster & Knook, 1982). In the presence of shunting, the sensitivity of the assay would determine the time at which endotoxin could be detected in hepatic venous blood.

Four other studies have investigated endotoxin removal when presented via the portal vein. Mori et al. (1973) demonstrated that endotoxin appeared to be less toxic to rats (using death as the measure of toxicity) when injected into the portal system than when injected systemically. Ruiter et al. (1981) compared the injection of a high sublethal dose of ^{51}Cr-labeled E.coli O26:B6 endotoxin (3 mg) into male Wistar rats (200-250 grammes body weight) with a ten fold lower dose (0.3 mg) injected either systemically or into the portal system. At the higher dose, only 5% of the injected dose was found in the liver at 30 minutes after systemic injection while 28% of the endotoxin injected into the portal system had localised there. At the lower dose 35% of the systemically administered endotoxin and 45% of that injected into the portal system, could be accounted for. At the higher dose, liver radioactivity fell. At the lower dose, there was a fall in blood radioactivity over about an hour, after which it began to rise again. Since this rise was associated with a progressive fall in liver radioactivity, it was suggested that it represented release of ^{51}Cr from endotoxin in the liver and that the mechanism of endotoxin clearance might well differ according to the magnitude of the injected dose. Carvana et al. (1984) infused 6 µg/ml/min of E.coli O26:B4 endotoxin (Difco) into the portal vein of ten adult mongrel dogs (18-22 Kg body weight) while maintained under pentabarbitone anaesthesia and mechanically ventilated. Endotoxin was assayed in hepatic venous blood using an immuno-radiometric technique which was specific for the endotoxin preparation used and which had a detection limit of 1 ng/ml. Systemic endotoxaemia occurred in all animals by 100 minutes. From their data, the mean extraction efficiency of the liver was calculated to be approximately 0.72 µg/gram liver/hour. Portal vein plasma flow (PvPF) reached its nadir at 300 g infused (50') but interestingly, systemic blood pressure and portal wedge pressure did not reach their nadir until approximately 540 µg had been infused (90 min).

In the most recent study (Yamaguchi et al., 1982), extending the studies of Mori et al. (1973), the endotoxin assay was based upon lethality in lead acetate treated male Sprague-Dawley rats (250-300 g body weight). All animals received 5 mg of lead acetate via the dorsal vein of the penis immediately before a femoral vein infusion (experimental animals had the inferior vena cava draining into the portal vein) of E.coli O55:B5 (Difco) endotoxin at various doses over one hour.

Three groups of animals were studied:

1. No surgery (controls) - 129 animals
2. Reversed Eck fistula (inferior vena cava anastomosed to portal vein so that a femoral vein injection reached the liver via the portal vein) - 78 animals
3. Sham operated (controls) - 149 animals.

Mortality from surgery was negligible, though only survivors at 24 hours following surgery were used. Following the one hour infusion, the femoral vein was ligated and the animals returned to individual cages and lethality assessed over 48 hours (though all non-survivors died within 24 hours). A dose-mortality curve could be constructed from the control results and the results of reversed Eck fistula (REF) animals could be related to this curve. For example, mortality in REF animals receiving 1 µg of endotoxin/100 g body weight approximated to that in sham operated animals receiving 15 ng/100 g body weight. It was then extrapolated that approximately 15 ng of the infused endotoxin failed to be cleared and that the liver extracted 985 ng. Likewise, the same calculations can be performed with the 2, 3 and 4 mg doses (see Table 7.2).

TABLE 7.2 RELATIONSHIP BETWEEN ADMINISTERED ENDOTOXIN DOSE AND LIVER EXTRACTION EFFICIENCY IN MALE SPRAGUE-DAWLEY RATS
(after Yamaguchi et al., 1982)

Infused dose (µg/h)	Spillover (ng/h)	Liver Sequestration (ng/h)
1	15	985
2	35	1965
3	190	2810
4	825	3175

These results follow saturation kinetics and therefore a Lineweaver-Burke plot allows calculation of the Vmax which is approximately 1.5 µg/gram liver/hr, a result in substantial agreement with that obtained by Carvana et al. (1984) in dogs.

In summary, these studies suggest that endotoxin removal is much more efficient when it is adminstered via the portal vein and that the *in vivo* capacity for endotoxin removal in two species whose sensitivity to the lethal effects of endotoxin differs by a factor of about 40, *is very similar*. This leads naturally into a discussion on how endotoxin toxicity and removal are related and how they may be modified.

7.1.7 Mechanisms of Endotoxin Toxicity

Many of the consequences of acute exposure to endotoxin resemble the changes seen in septic shock and may be summarised as follows:

Neutropaenia
Thrombocytopaenia
Disseminated intravascular coagulation
Changes in blood flow regulation
Respiratory alkalosis (transient)
Metabolic acidosis (persistent)
Hyperglycaemia (transient)
Hypoglycaemia (sustained)
Skeletal muscle catabolism
Blood and liver lipid changes
Changes in body temperature
Shock (cardiovascular collapse)
Death

Large quantities of injected endotoxins are taken up by mononuclear phagocytes (especially the hepatic Kupffer cells). Great interest has focussed on the role of mediators in the pathophysiology of endotoxic and septic shock (Lefer, 1985; Filkins, 1985; Ford-Hutchinson, 1985) since mononuclear phagocytes are known to produce mediators upon phagocytic stimulation. Indeed, Fine argues a role for endotoxins in all forms of shock. Attempts at intervention with specific inhibitors of some of these mediator substances has produced only modest success (Altura, 1980), so the discussion here will focus upon two aspects, namely carbohydrate metabolism and the production of leukotrienes, since these may be of particular relevance to the experiments reported later in this chapter.

7.1.7(a) Hypoglycaemia

Hypoglycaemia following endotoxin injection is well documented in many species (for review, see Spitzer, 1984). In man, prolonged hypoglycaemia is associated with damage to parenchymal liver cells (Soler & Khardori, 1985). During the hypoglycaemic phase, hepatic glucose production is increased over control values but still fails to keep pace with glucose utilisation in the liver and other tissues. Total liver blood flow (in these studies) is well maintained, though the contribution from the hepatic artery increases in proportion to the fall in blood flow through the portal vein (Naylor & Kronfeld, 1985). The rate of oxygen delivery is unlikely to be responsible since, with the increased contribution from the hepatic artery, the oxygen tension actually increases. Nevertheless, it is possible that cells which have been hypoxic may be sensitised to oxygen-induced damage. Such temporary hypoxia may result from temporary occlusions at the level of the sinusoid. At the Third International Kupffer Cell Symposium, McCuskey, presented clear evidence

that such temporary occlusions at the level of the sinusoid do occur after endotoxin administration.

Insulin causes increased glucose utilisation and a number of relevant observations have been made in this regard (Filkins et al., 1981).

1. Increased insulin release by the pancreas induced by endotoxin (early)
2. Direct insulin-like activity of endotoxin
3. Production of a monokine(s) with insulin-like activity

Glucocorticoid resistance could also explain inadequate gluconeogenesis since the enzyme phospho-enol pyruvate carboxykinase (PEP-CK) is normally induced by glucocorticoids and is a rate-limiting step in gluconeogenesis. A failure of glucocorticoids to induce PEP-CK in endotoxin poisoned animals has indeed been reported (Berry & Huff, 1984). Hormone-receptor binding and subsequent transfer to the nucleus are unimpaired but levels of PEP-CK mRNA are greatly reduced. This effect is believed to be brought about by the monokine; glucocorticoid antagonistic factor (GAF). Injection of this substance sentitises animals to endotoxin, heat and cold by inducing what is effectively a functional adrenalectomy. GAF appears to be produced in large amounts in response to endotoxin and can be detected in the blood of endotoxin-treated mice. Studies using hydrocortisone induction of PEP-CK in Reuber H35 hepatoma cells clearly demonstrated that it is not a direct endotoxin effect but is mediated by GAF (see Berry & Huff, 1984).

7.1.7(b) Leukotrienes

Leukotrienes are produced from arachidonic acid along the 5-lipoxygenase pathway. Leukotriene C_4 (LTC_4) is substituted with glutathione and leukotrienes D_4 (LTD_4) and -E_4 (LTE_4), with short peptide chains. They can be produced in many cell types including mast cells, neutrophil and eosinophil granulocytes and mononuclear phagocytes (Ouwendijk, 1985). Leukotriene B_4 (LTB_4) appears to be very important in the induction of inflammatory infiltrates, in stimulating natural killer cell activity and in inhibiting T lymphocyte proliferation. LTC_4 and LTD_4 can induce phospholipase A_2 (and thereby enhance the supply of arachidonic acid for further production of eicosanoids) and have profound effects on smooth muscle (constriction) through a specific receptor interaction. Many of the features of endotoxic shock (eg myocardial depression, increased vascular permeability and leukocyte adhesion) can be reproduced by the injection of leukotrienes (Hagmann et al., 1985).

Many of the cell types which produce leukotrienes can also degrade them. Granulocytes do so by oxidation (utilising the peroxidase-hydrogen peroxide-halide system or by generation of the hydroxyl radical) or by sequential peptide bond cleavage. Mononuclear phagocytes can also degrade them by oxidation involving myeloperoxidase and hydrogen peroxide. This pathway can be inhibited by azide

and catalase but not by superoxide dismutase (SOD). Oxidation can also take place in hepatocytes via cytochrome P450 (Neill et al., 1985). In rodents and lagomorphs, hepatobiliary elimination with partial or complete cleavage of amino acids is the major route of eliminatoin for amino acid-substitutes leukotrienes (Hagmann et al., 1985) and this route of elimination is inhibited by endotoxin (thus potentiating their effects). In man, the kidney also, is a major organ of elimination.

There is evidence that leukotrienes may be involved in endotoxin-induced liver injury (Keppler et al., 1985). The major hepatic lesions induced by endotoxins are hepatocellular necrosis and inflammatory cell infiltrates (Hirata et al., 1980; Nordstoga & Aasen, 1979; Sato et al., 1982). Essential fatty acid depleted rats (depleted of arachidonic acid) are resistant to endotoxin, and also resistant to sensitisation to endotoxin by intravenous particulate by glucan (Table 7.3). Sensitisation to both phenomena is restored by intra-peritoneal ethyl arachidonate 4 and 1 days before LPS (Cook et al., 1982). Liver injury is enhanced when the endotoxin is injected with an inhibitor of hepatocellular RNA synthesis such as D-galactosamine or alpha-amanitin. Administration of inhibitors of leukotriene synthesis aborts the sensitising action of these substances and the severe zonal necrosis, isolated parenchymal cell necrosis and inflammatory infiltrates are of only comparable severity to what may be seen with endotoxin alone in unsensitised animals. Neither D-galactosamine nor alpha-amanitin produce liver injury in the absence of endotoxin. These findings suggest that leukotrienes may play a role in the pathogenesis of the liver injury following endotoxin administration. However, inhibitors of leukotriene synthesis do not prevent death in endotoxic shock in all studies; e.g. McKechnie et al. (1985), who showed that BW755C (an inhibitor of both the cyclooxygenase and lipoxygenase pathways) completely aborts changes in plasma prostanoids but does not prevent mortality.

In conclusion, it may be stated that leukotrienes are produced following the administration of endotoxin and that leukotrienes may be responsible for at least some of the secondary effects of endotoxins. It is also clear that other factors may be decisive in predicting the outcome of endotoxin administration.

7.1.8 Experimental Modulation of Endotoxin Removal and Toxicity

This topic is difficult to address since it is frequently impossible to separate the sequelae of endotoxin injection from the sequelae of shock. This is further complicated by Fine's suggestion that endotoxaemia is an accompaniment of all forms of shock. Intervention studies with a variety of therapeutic agents are similarly complicated.

The notion that the state of activity of the reticuloendothelial system (RES) could be a major determinant of the expression of endotoxin toxicity, followed the experiment of Beeson in 1947 in which it was observed that rabbits rendered tolerant to both the pyrogenic and lethal effects of endotoxins could be resensitised by administering large doses of colloidal substances which rendered the

RES dysfunctional. With the knowledge that the liver was the major organ for endotoxin localisation and detoxification, it soon came to be believed that RES stimulation would ameliorate endotoxin activity while RES depression would exacerbate. This concept soon became untenable when numerous studies showed that there was no simple relationship between RES activity (as assessed by colloid clearance) and the state of endotoxin sensitivity or resistance (see Munson & Regelson, 1971; Filkins & Buchanan, 1972; Trejo et al., 1972; Lazar et al., 1977; Di Luzio et al., 1980). These studies are summarised in Table 7.3

TABLE 7.3 SUBSTANCES MODULATING RETICULOENDOTHELIAL SYSTEM (RES) ACTIVITY AND ENDOTOXIN SENSITIVITY

Substance	RES activity	Sensitivity to endotoxin
Bacille Calmette-Guèrin	++	+
Muramyl dipeptides	++	+
Corynebacterium parvum	++	+
Zymosan	++	+
Glucan (soluble)	?	sl -
Glucan (particulate) iv	++	+
Glucan (particulate) ip	?	sl -
Methyl palmitate	--	-
Cholesterol oleate	--	-
Thorotrast	-	+
Trypan blue	-	+
Saccharide iron oxide*	-	+
Carbon	-	+
PCN	±	±
DES	++	±
Actinomycin D*	?	+
Lead acetate	-	+
Vitamin A excess *	?	+ BM
Pyran copolymer (early)	-	+
Pyran copolymer (late)	+	+

abbreviations: iv = intravenously; ip = intraperitoneally;
PCN = pregnenolone-16 alpha-carbonitrile
DES diethyl stilboestrol
* = substances which enhance production or reduce detoxification of free radicals
BM = only bone marrow (BM) cytotoxicity was tested
sl = slight + = enhance - = reduce ± = equivocal
++ = greatly enhance -- = greatly reduce

Unfortunately, very few of the studies summarised in Table 7.3 specifically assessed endotoxin clearance. In one study by Filkins and Buchanan (1973) it was clearly demonstrated that lead acetate had no effect upon endotoxin removal in vivo or upon endotoxin detoxification by liver homogenates in vitro. These authors went on to suggest that the most likely explanation is that lead salts lead to an enhancement of endotoxin toxicity by altering metabolic responses during endotoxaemia.

Experiments involving endotoxin administration should take into account the possibility that some of the changes may be part of the shock syndrome. Altura (1980) separates all shock syndromes into three phases on the basis of the haemodynamic changes:

1. Compensatory
2. Stagnant hypoxia
3. De-compensatory.

The use of vaso-active drugs as shock therapy has given generally unsatisfactory results but this may be because the agents used lacked selectivity and possessed additional properties which are inappropriate or even deleterious to a particular phase of shock. The matching of specific drug actions to the particular phase of shock may give improved results.

Regardless of the nature or aetiology of the shock state, reticuloendothelial system (RES) depression (as assessed by colloid clearance) parallels the degree of shock or trauma. Such RES drepression persists until death in non-survivors. Survivors exhibit recovery of RES activity within 24-48 hours and frequently display enhanced activity over that seen in controls. Changes in plasma fibronectin (an opsonic glycoprotein) follow the changes in RES activity with a lag phase (see Chapter VI). The RES activity has usually returned to normal by day 4 while plasma fibronectin levels are still elevated on day 5. This suggests that using plasma fibronectin determinations as a predictor of RES activity is not warranted.

Administration of near lethal doses (1 mg/100 g body weight) of a phenol-extracted endotoxin preparation from Salmonella abortus equi (smooth form) results in RES depression of colloid clearance over the first 12 hours or so and is followed by RES stimulation reaching a maximum between days 3 and 5. It was not shown whether the depressed RES activity was associated with saturation of uptake capacity, cell death or both. At the time when RES activity is enhanced, there is increased DNA synthesis within the hepatic sinusoidal lining cells (as assessed by autoradiography of liver slices following exposure to tritated thymidine in vivo; Gospos et al., 1977). Altura (1980) argues that RES stimulation is a rational therapy for shock states in man. This suggestion merits a note of caution since some RES stimulants actually enhance the toxic effects of endotoxins (Table 7.3).

The efficacy of the prophylactic use of some steroid hormones in shock states in both humans and experimental animals is well documented (Altura, 1980). For example, large doses of oestradiol in rodents not only protect against several types

of shock but also prevent the early depression in RES phagocytic activity. Glucocorticoids are also effective when given prophylactically. Not only do they prevent the early depression of RES phagocytic activity, but actually enhance the clearance of endotoxin. The mechanism of the protective action of these steroid hormones is not simply to maintain RES phagocytic activity, and a number of other possibilities exist:

Metabolic actions (see sections 1.5 and 7.1.7)
Inotropic action on the heart
Alpha-adrenergic blockade
Functional antagonism between glucocorticoids and vasoactive substances
Membrane stabilistaion
Immunological changes

The levels of a number of eicosanoids are known to be elevated following endotoxin administration (Ouwendijk, 1985; McKechnie et al., 1985). It has been suggested that inhibitors of eicosanoid production may modify several features of experimental endotoxin shock (Parratt & Sturgess, 1976). Among the varied actions of prednisolone, it is able to selectively block the production of some eicosanoids (eg 6-Keto PGF1α) but has no effect on the production of others (eg Thromboxane B2). On the other hand, BW755C (a blocker of both prostaglandin and leukotriene synthesis) completely prevents the endotoxin-induced increase in the urinary and plasma levels of breakdown products of thromboxane and prostacyclin and presumably, therefore, of their precursors (and it probably also blocks the lipoxygenase pathway, though this was not measured directly) but failed to modify the metabolic changes associated with endotoxaemia or lethality. It may be concluded that a complex balance exists between the effects of different eicosanoids, some of which may be beneficial and some harmful. Nonetheless, other changes must underly the metabolic changes and lethality induced by endotoxins.
Another interesting possibility for the mediation of some harmful effects of endotoxin is the generation of highly reactive oxygen species (for review, see Halliwell-Gutteridge, 1985). The major forms of such active oxygen species are superoxide and its conjugate acid, the hydroperoxy radical; singlet oxygen; the hydroxyl radical; and hydrogen peroxide. Cells possess a number of enzyme systems responsible for the rapid containment of these substances (Cerutti, 1985). Iron salts are essential for the formation of free radicals and the administration of iron salts may exacerbate a number of inflammatory disorders (bacterial infections, rheumatoid disease). Likewise, Actinomycin D (a potent sensitising agent to endotoxins) is slowly reduced inside cells to yield an intermediate which can generate superoxide. In vitro studies have also shown that both retinoic acid and retinol to be potent generators of superoxide as well as being free radical scavengers. Results were presented from the Institute for Experimental Gerontology at the recent Third International Kupffer Cell Symposium (Strasbourg, France, 1985) which showed that hypervitaminosis A markedly sensitises rats to

the liver injury produced by endotoxins. Vitamin E is an excellent free radical scavenger and singlet oxygen quencher and a good in vitro anti-oxidant (Pekkanen et al., 1983). Pre-treatment with vitamin E ameliorated the liver injury induced by endotoxin though the possibility remains that this effect is ultimately due to an inhibition of eicosanoid production. One recent study utilising a selective hydroxyl radical scavenger failed to demonstrate any protection against endotoxin-induced lung injury (Wong et al., 1985).

The next possibility to be discussed will relate to the immune system, endotoxin sensitivity and protection against endotoxin effects. It has been known for some time that the spleen exerts a permissive role on the phagocytic activity of the entire RES (Altura, 1978). This effect may be mediated through the phagocytosis promoting peptide, tuftsin. Evidence also exists that T lymphocytes can modulate the sensitivity of macrophages to endotoxin (Rosenstreich & Vogel, 1980). The effect appears to be mediated by a lymphokine and this substance can restore the sensitivity to endotoxin of macrophages derived from C3H/HeJ mice (an endotoxin-resistant strain). No-one has yet characterised this lymphokine and the possibility that it may be similar (or even identical) to tuftsin is worthy of consideration. This observation is extremely important if the notion that mononuclear phagocytes are the primary determinants of an organism's sensitivity to endotoxins is valid. The therapeutic potential of these lymphokines has not been investigated.

Lastly, perhaps the most direct way of modulating endotoxin effects in vivo is by means of specific antibody. Some of the evidence has already been reviewed in chapter 1. The approach which has attracted most interest is that of passive immunisation. Most antibodies that have been used to date were raised against the core glycolipid of a rough mutant (J5) of E.coli (reviewed by Braude & Ziegler, 1983). Antibodies were either raised in human volunteers or were manufactured in vitro using human-human hybridomas. Such antibodies confer a wide spectrum of protection against many species of gram negative bacteria and their endotoxins, can be used successfully for the prophylaxis of gram negative septicaemia and shock and may even reverse the progression of endotoxic and gram negative septicaemic shock (Braude & Ziegler, 1983).

7.1.9 Sensitivity to Endotoxins and the Specific Pathogen-Free and the Germ-Free States

The concept that the intestinal flora is important in the regulation of mucosal immunity was proposed in Chapter 1. It is also believed that the enteric flora may be important as a determinant of sensitivity (or resistance) to endotoxin-induced lethality. Schaedler and Dubos (1961, 1962) performed experiments using a mouse strain (NCS) which had no E.coli in its enteric flora and the strain from which it was derived (SS) which did have E.coli in its enteric flora. The NCS mice were six times more resistant to the lethal effects of endotoxin than were the SS mice.

When E.coli was introduced to the enteric flora of the NCS mice, they displayed the same sensitivity to endotoxin to what is observed in the SS mice. Studies with truly germ-free mice have given conflicting results. Two studies (Landy et al., 1961; Abernathy & Landy, 1967) found no difference in sensitivity between germ-free mice and their conventional counterparts, to the lethal effects of endotoxin. This contrasts sharply to the increased resistance found in germ-free mice in three other studies (Jensen et al., 1963; Sacquet & Charlier, 1965; Kiyono et al., 1980). Germ-free rodents are resistant to thermal injury (Markley et al., 1969) but not to haemorrhagic shock (McNulty & Linares, 1960; Zweifach et al., 1958).

Abnormalities have been recorded in macrophages isolated from germ-free rodents (Heise & Myrvik, 1966; Jungi & McGregor, 1978; Morland et al., 1979; Starling & Balish, 1981). Macrophages are fewer in number, have impaired phagocytosis mediated by the complement receptor, have reduced chemotaxis and lower lysosomal enzyme activity. When germ-free rats are returned to the conventional state, there is an increase in the number of alveolar macrophages and their lysosomal enzyme activity is enhanced (Starling & Balish, 1981).

7.2 MATERIALS AND METHODS

7.2.1 Endotoxin

A phenol-water extract of E.coli O26:B6 (Sigma Chemical Company, St. Louis. MO, USA; lot number 93f-4041) was used throughout. When required, this preparation was labeled with ^{51}Cr, after the method of Braude (Braude et al., 1955). Briefly, 17.5 ml of endotoxin solution (27.5 mg) was added to 1 mCi of $Na^{51}CrO_3$ and incubated at 37°C for 24 hours. This was followed by dialysis against repeated changes of distilled water and finally physiological saline until there was no further loss of radioactivity into the dialysate. The final preparation had 95% of the radioactivity precipitable by absolute ethanol (indicating that it was associated with the endotoxin). The specific activity of the final preparation was 5×10^3 cpm/mg. The labeling procedure did not affect the ability of the endotxoin to activate Limulus amoebocyte lysate (see later) since both the labeled and unlabeled endotoxins exhibited an activity of approximately 2.5×10^6 endotoxin units (EU) per milligram.

7.2.2 Animals

Rats were obtained from the Institute's ageing colony and were housed under clean conventional conditions before and during experiments. For details about this ageing colony, the reader is referred to Burek (1978) and Van Zwieten (1984). All animals were allowed free access to food and water and were maintained at an

ambient temperature of 19-21°C unless otherwise stated. A total of 111 animals of differing age and sex and from two different strains (BN/BiRij and WAG/Rij) were used. The survival characteristics of these strains are shown in Table 7.4.

TABLE 7.4 SURVIVAL CHARACTERISTICS OF BN/BiRij AND WAG/Rij RATS

Strain	Sex	Number of animals	Age (mo) at survival points			
			90%	50%	10%	Maximum
BN/BiRij	M	286	20.8	30.5	35.5	39.9
BN/BiRij	F	885	22.4	31.8	37.6	41.3
WAG/Rij*	M	276	20.1	27.2	33.5	36.5
WAG/Rij	F	984	24.5	33.2	39.2	44.5

* not used in these experiments

Further details of animals injected intravenously with endotoxin in the various experiments are summarised in Table 7.5.

Following intravenous injection with endotoxin, all animals were maintained at an ambient temperature of 19-21°C except for a group of five, female, BN/BiRij rats aged 24 months which were housed in a heating cabinet where the ambient temperature could be controlled so as to prevent any fall in body temperature ("Temperature Clamp"). Furthermore, animals were allowed free access to food and water and were closely observed for the duration of the experiment. Investigations were performed as indicated in Table 7.5. Animals were either killed "in extremis" by exsanguination under ether anaesthesia or were killed in the same way at either 24 or 48 hours following endotoxin administration (as indicated).

The histopathological investigations reported here were carried out on 33 female BN/BiRij rats of different ages. After exsanguination, the liver, spleen and kidneys were removed and fixed in 4% buffered formalin and later processed for routine histopathological assessment by light microscopy. All histological interpretations were carried out by the same experienced pathologist.

7.2.3 Procedures

Endotoxin injection: This was performed by one of two methods: either external jugular vein injection or tail vein injection. All animals were injected into the jugular vein except for those receiving the very low dose of endotoxin

TABLE 7.5 SUMMARY OF EXPERIMENTS (For explanation, see text)

Exp.	Strain	Nr.per age group	Sex	Age (mo)	ET dose (mg/100 g bw)	Gamma counting	LAL test	T-A	Glucose	Temperature	Histology	Endpoint
1.	BN/BiRij	3	F	6	10	-	-	+	+	+	-	48 hr
2.	BN/BiRij	5	F	3,6,24,36	4	+	-	+	-	-	+	24 hr
3.	BN/BiRij	5	F	3,6,24,36	2	+	+	+	-	-	+	24 hr
4.	BN/BiRij	5	F	6,24	1	+	-	+	-	-	-	24 hr
5.	BN/BiRij*	5	F	24	1	-	-	+	+	+	-	48 hr
6.	BN/BiRij	5	M	6,24	1	-	-	+	+	+	-	48 hr
7.	WAG/Rij	5	F	6,24	1	-	-	+	+	+	-	48 hr
9.	BN/BiRij	5	F	18	1	-	-	+	+	+	-	48 hr
10.	BN/BiRij	2,3	F	6,24	0.0001	-	-	+	+	+	-	48 hr

bw = body weight
T-A = transaminase measurement
+ = performed and reported here
- = not performed or not reported here
* = temperature "clamp"

F = female; M = male; Exp. = experiment;
ET = endotoxin; LAL = Limulus amoebocyte lysate

(0.0001 mg/100 g body weight). Both procedures were performed under light ether anaesthesia. For external jugular vein injection, a lateral longitudinal incision was made in the neck and the vein exposed by blunt dissection. The injection was made through the pectoralis muscle with a 27G needle. Before tail vein injection, animals were kept under a heat source for a few minutes to ensure maximal vasodilatation. The injection was made percutaneously with a 25G needle.

Blood sampling: With the animal still under light ether anaesthesia, the tail vein was transected by a lateral incision near to the tip of the tail. The frequency of sampling depended on the experiment and this is made clear in the results section. For the disappearance curves of endotoxin, 150 μl of blood were collected into heparinised glass capillaries, sealed at one end and kept on ice until spun in a microhaematocrit centrifuge and the plasma drawn off. Samples for glucose estimation were collected directly into commercially available glass tubes containing EDTA (as anticoagulant) and NaF (as a metabolic inhibitor). Samples were kept below 4°C and assayed within 24 hours. Samples for transaminases were obtained "peri mortem" when the animal was exsanguinated through the inferior vena cava. Blood was allowed to clot in plastic tubes and then the serum was drawn off and stored at -20°C until the assay was performed.

Temperature measurement: This was done by inserting a lubricated thermistor probe at least 3 cm into the rectum and 30 seconds were allowed to elapse for the instrument to stabilise.

Assay procedures: The Limulus amoebocyte lysate (LAL) test was performed as described in Chapter II, section 2.8. The serum glutamate-oxaloacetae transaminase (SGOT) and the serum glutamate-pyruvate transaminase (SGPT) were measured using reagent sets obtained from Boehringer-Mannheim. The blood glucose concentration was measured in the laboratory of the Stichting Samenwerking Delftse Ziekenhuis using the glucose dehydrogenase method (Merck).

Radioactivity was measured in 20 μl plasma samples by counting for five minutes in a gamma scintillation counter. Appropriate standards and blanks were included in each run. Results were eventually expressed as counts per minute per millilitre of plasma (cpm/ml).

7.2.4 Endotoxin Absorption from the Gut

This experiment employed techniques reported by Ouwendijk (1985). Two groups of nine female BN/BiRij rats (aged 6 and 24 months) were used. Animals were placed under halothane anaesthesia and a midline laparotomy performed. To assess endotoxin absorption from the small intestine, a loop of approximately 15 cm length was chosen immediately distal to the duodenum. Two loose ligatures were placed approximately 15 cm apart, taking care not to damage the vasculature. A small incision was then made proximal to the first of the loose ligatures and a cannula (1 mm external diameter) was passed through so that the tip lay in the gut lumen between the two loose ligatures. The two ligatures were then tightened

and the cannula secured by means of a third ligature just proximal to its site of entry. Two millilitres of ^{51}Cr-labeled endotoxin (0.5 mg/ml) were then instilled into the "in situ" loop, immediately followed by 0.25 ml of air. The cannula was sealed by heating and the abdomen closed in layers with clips to the skin. Animals were allowed to recover and were later sacrificed under ether anaesthesia 2.5 hours after the introduction of endotoxin into the loop. Blood was obtained from the inferior vena cava and portal veins by aspiration. The isolated loop was removed intact and the length measured after attaching a standard weight to one end. The remaining gut, together with liver, spleen, kidneys, adrenals, heart and lungs, was removed and each organ (or group of organs) placed into plastic vials and radioactivity counted over 10 minutes in a gamma scintillation counter. The radioactivity in the blood and organs was expressed as a precentage of the total radioactivity in these organs plus the isolated loop. Absorption was calculated as "g per cm of gut per 2.5 hours.

To assess endotoxin absorption from the colon, the terminal 8 cm was chosen. The procedure outlined above was followed except that the gut was gently irrigated with 0.154 M NaCl and only 1 ml of the endotoxin solution was introduced into the loop.

7.2.5 Data Handling

Plasma disappearance curves and mortality curves were constructed using the Institute's Data General main frame computer. Plasma disappearance curves were further analysed by plotting regression lines for measurements between the times 2 and 10 hours. All these regression lines were acceptable at the 5% level of probability. Plasma half life (t½) could then be calculated from these regression lines. All data between groups were compared by means of non-parametric statistics (Mann-Whitney U test) and significance was set at the 5% level of probability.

7.3 RESULTS

7.3.1. Clinical Features

Both young and old animals reacted initially in the same way following an endotoxin injection. In the 1-4 mg/100g body weight dose range, all animals displayed diminished activity and adopted a hunched posture which would tend to reduce the surface area/volume ratio. Epiphora, pilo-erection and shivering were all observed. As time progressed, diarrhoea was occasionally seen in both age groups and haematuria in some older animals. By about 90 minutes, blood seemed hypercoagulable and viscous. This state persisted in the young rats throughout the experiment but had subsided in the 24- and 36-month-old rats by 4-6 hours. After about 4 hours had elapsed, older animals tended to uncurl themselves (thus increasing the surface area/volume ratio), became increasingly lethargic and tended

to lie down rather than to stand on all four limbs. At this time, the older animals were palpably cold. These features were not observed in young animals unless they had been injected with a very large dose of endotoxin (10mg/100g body weight). As time progressed further, the older animals lapsed into coma and generalised convulsions were sometimes seen, especially in the 36 month old animals. Most young animals survived endotoxin injection in the dose range 1-4 mg/100g body weight. When these animals did die, the time course from first appearing ill to death was very rapid.

7.3.2 Endotoxin Disappearance from Blood

Plasma disappearance curves derived from a 6 and from a 24 month old rat (injected with 2mg/100g body weight) are illustrated in Figure 7.3 (experiment 3). The elimination half life (from experiment 2-4) is consistently longer in 3-month-old animals than in 6-month-old animals injected with either 2- or 4 mg of endotoxin per 100 g body weight. In animals injected with 1-, 2- or 4 mg/100 g body weight, the 6-month-old animals cleared endotoxin significantly more rapidly than did the 24-month-old animals (Tables 7.6 and 7.7). Only in animals injected with 4 mg/100 g body weight of endotoxin did the oldest animals (aged 36 months) clear the endotoxin more rapidly than any other age group.

A very interesting finding from experiment 3 is that the Limulus amoebocyte lysate (LAL) test gave significantly shorter plasma elimination half lives than were obtained when radioactivity was followed. It must be stressed that the two tests give different information. The LAL test indicates an activity of endotoxin while radioactivity indicates an amount. It is certainly possible that LAL activity could be reduced following some event in the plasma or by the most "active" forms of endotoxin being removed preferentially. It is also important to point out that the elimination half life determined by following radioactivity could be prolonged by release of ^{51}Cr from endotoxin which has already been cleared. It is possible (though considered unlikely by the author!) that the 36-month-old animals giving a short plasma elimination half-life do so because ^{51}Cr has not been cleaved from the endotoxin and therefore not released into the circulation. A further important observation for the interpretation of these results is that intravenously injected $Na^{51}CrO_3$ has a plama half life of about 8 hours, but this does not increase as a function of age (G.J.M.J. Horbach, personal communication).

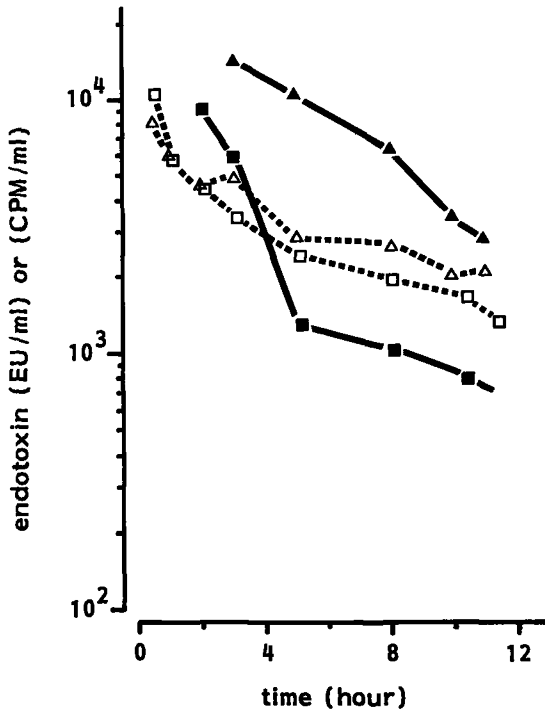


Fig. 7.3 Plasma disappearance of intravenously injected E.coli O26: B6 endotoxin in a 6 month old (squares) and a 24 months old (triangles) BN/BiRij rat followed by both the LAL test (closed symbols) and radioactivity counting (open symbols).

TABLE 7.6 COMPARISON OF PLASMA HALF-LIFE OF E.COLI O26:B6 ENDOTOXIN INJECTED INTO FEMALE BN/BiRij RATS OF DIFFERENT AGES (2 mg/100 g body weight)

Age (mo)	Half-life (hrs) (Radioactivity)	Half-life (hrs) (LAL test)
3	8.22 (± 0.63)	2.89 (± 0.20)
6	7.11 (± 0.47)	3.29 (± 0.19)
24	10.51 (± 0.48)*	4.73 (± 0.30)*
36	11.73 (± 1.77)**	4.75 (± 0.92)

statistical analysis by the Mann-Whitney U test
results presented as mean (± standard error)

* : significantly different from 3 and 6 month groups ($p < 0.05$)
** : significantly different from 6 month group ($p < 0.05$)
mo : months

TABLE 7.7 COMPARISON OF PLASMA HALF-LIFE OF E.COLI O26:B6 ENDOTOXIN INJECTED INTO FEMALE BN/BiRij RATS OF DIFFERENT AGES (1 or 4 mg/100 g body weight)

Age (mo)	Dose (mg/100 g bw)	Half-life (hrs) (radioactivity)
3	4	10.11 (± 1.34)
6	4	9.21 (± 0.56)
24	4	11.02 (± 0.77)*
36	4	8.73 (± 0.53)
6	1	7.45 (± 0.41)
24	1	12.13 (± 1.55)**

statistical analysis by the Mann-Whitney U test
results presented as mean (± standard error)

* : significantly different from the 6 and 36 month groups ($p < 0.05$)
** : significantly different from the 6 month group ($p < 0.05$)
bw : body weight

7.3.3 Endotoxin-induced lethality

All animals aged 24 months and older died following the injection of endotoxin. The time course and dose-response relationship of this is illustrated for 24 month old female BN/BiRij rats in Figure 7.4. There is a clear relationship between the injected dose and the time which elapses between the first and last death in each group. There is also a clear relationship between the injected dose and the time at which the first animal died in each group. All animals died very quickly with the 4 mg dose (experiment 2) and to a lesser extent with the 2 mg dose (experiment 3). The time course was somewhat more prolonged with the 1 mg dose (experiment 5). The 36 month old animals displayed a very similar response to that seen in the 24 month old animals. With the 4 mg/100 g body weight dose, the 36 month old animals all died between 5 and 6 hours and with the 2 mg/100 g body weight dose, they died between 5 and 7 hours. Only sporadic deaths were observed in 3 and 6 month old rats (two deaths in the whole series of experiments reported here).

This dramatically increased sensitivity to the lethal effects of endotoxin was apparently independent of sex and strain since it was also seen in 24 month old male BN/BiRij rats (experiment 6) and in 24 month old female WAG/Rij rats (experiment 7), though in the latter group, the time course of lethality for the group was somewhat more prolonged than was seen in BN/BiRij rats of either sex (Fig. 7.5).

The onset and time course of lethality in 6 month old female BN/BiRij rats injected with 10mg/100g body weight (experiment 1) and 18 month old female BN/BiRij rats (experiment 9) injected with 1 mg/100 g body weight can be deduced from the temperature curves illustrated in figures 7.6 and 7.9, respectively. The onset and time course of lethality for these 6 month olds was very similar to that seen in the 24 month old rats injected with 4 mg/100 g body weight. Eighteen month old animals showed only a 40% mortality over a much prolonged time course.

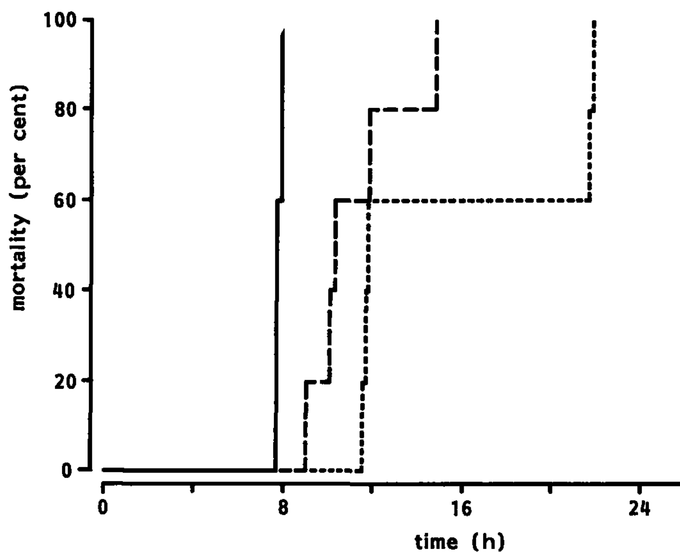
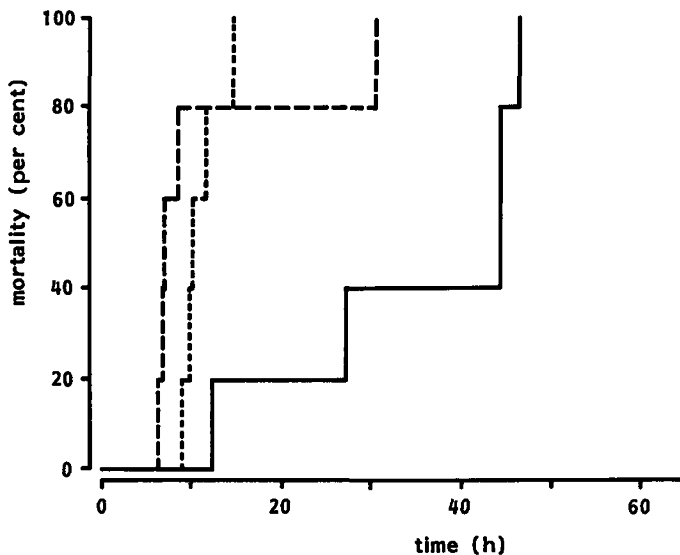


Fig. 7.4 Mortality curves for groups of 24 month old BN/BiRij female rats injected with 4 mg/100g bw (continuous line), 2mg/100g bw (broken line) and 1mg/100g bw (dotted line).



7.3.4 Body Temperature Control (experiments 6-10)

All animals that died following endotoxin injection showed a progressive fall in body temperature. Data for female BN/BiRij rats (aged 6 months) injected with 10mg/100g bw appear in Figure 7.6; for male BN/BiRij rats (aged 6 and 24 months) injected with 1mg/100g bw, in Figure 7.7; for female WAG/Rij rats (aged 6 an 24 months) injected with 1mg/100g bw, in Figure 7.8; and for female BN/BiRij rats (aged 18 months) injected with 1 mg/100g body weight, in Figure 7.9. It is apparent that the 18 month old female BN/BiRij rats which survived all started to lose heat after about 30 hours and this was associated with the animals becoming lethargic. It is believed (though not demonstrated) that these three animals would have died, had the experiment progressed longer than 48 hours. When two 6 month old and three 24 month old, female BN/BiRij rats were injected with 0.1 µg of endotoxin, no drop in body temperature was observed. Furthermore, within an hour of injection both groups had developed modest fevers of similar magnitude.

The basal body temperature did not vary markedly between the various groups or within those groups (Table 7.8) though WAG/Rij rats had significantly higher temperatures than did BN/BiRij rats and 6 month old rats had significantly higher temperatures than did the 24 month old rats of the same strain.

TABLE 7.8 BASAL BODY TEMPERATURE IN GROUPS OF RATS OF DIFFERING AGE AND STRAIN

Strain	Age	Basal temperature (mean, ± SD)
WAG female	6 months	37.44 (± 0.29)
BN male	6 months	37.06 (± 0.23)
WAG female	24 months	37.50 (± 0.31)
BN male	24 months	36.62 (± 0.55)

Fig. 7.5 Mortality curves for groups of 24 month old male BN/BiRij rats (dotted line), 24 month old BN/BiRij female rats (broken line) and 24 month old WAG/Rij rats (continuous line), injected with 1 mg/100 g body weight of endotoxin.

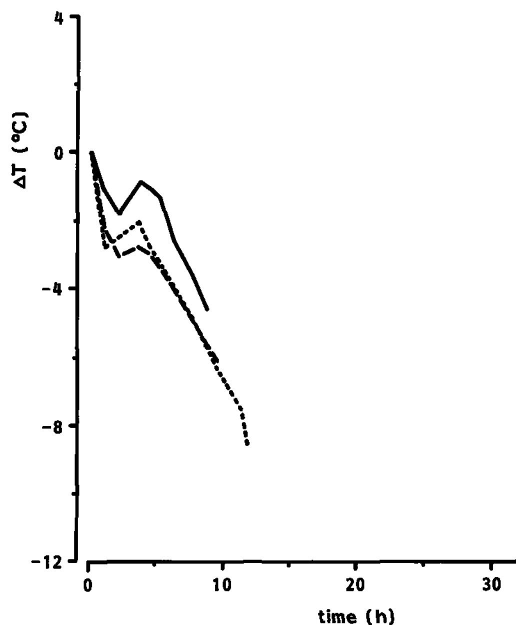


Fig. 7.6 Changes in body temperature compared with the basal body temperature in three BN/BiRij female rats (aged 6 months) injected with 10 mg/100 g bw of E.coli O26:B6 endotoxin.

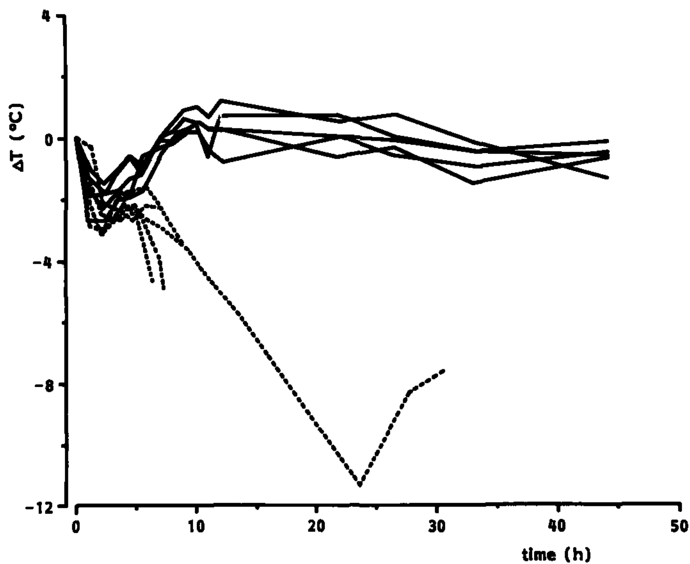


Fig. 7.7 Changes in body temperature compared with the basal body temperature in five male BN/BiRij rats aged 6 months (unbroken line) and five male BN/BiRij rats aged 24 months (broken line) injected with 1 mg/100 g bw of E.coli O26:B6 endotoxin.

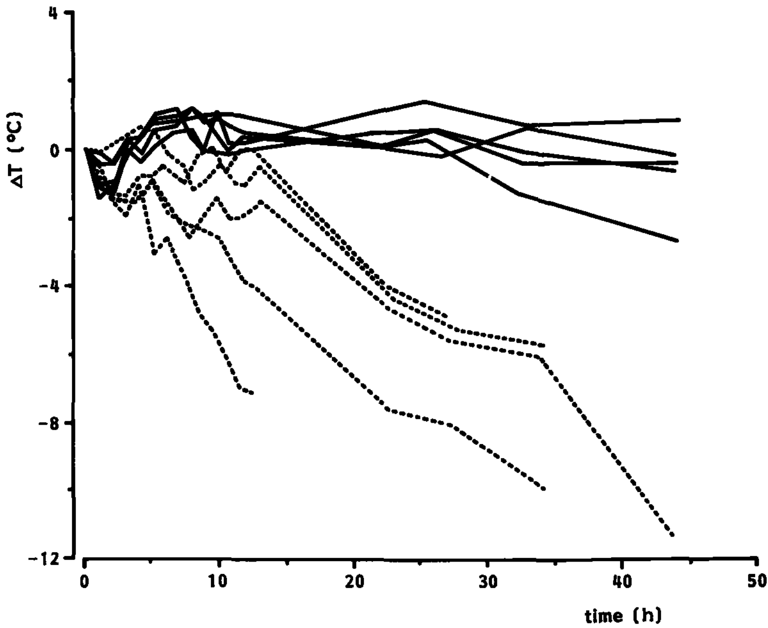


Fig. 7.8 Changes in body temperature compared with the basal body temperature in five female WAG/Rij rats aged 6 months (unbroken line) and five female WAG/Rij rats aged 24 months (broken line) injected with 1 mg/100 g body weight of E.coli O26:B6 endotoxin.

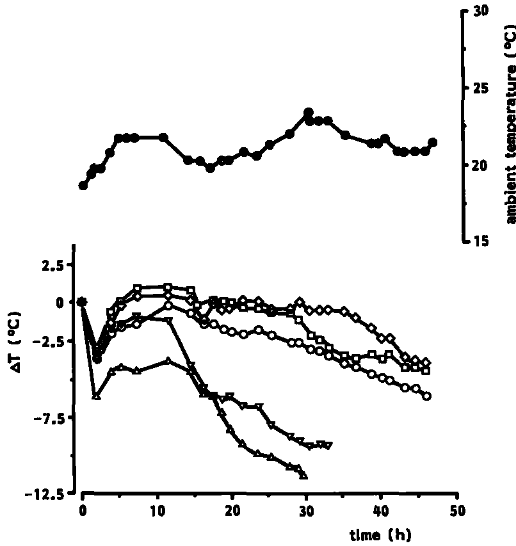


Fig. 7.9 Changes in body temperature compared with the basal body temperature in five female BN/BiRij rats aged 18 months, injected with 1 mg/100 g body weight E.coli O26:B6 endotoxin.

7.3.5 Histopathological Changes (experiment 2)

Organs from 33 animals were available for histological studies. No comment can be made about the time course over which the various lesions develop since the old animals were sacrificed at 24 hours.

Significant lesions were observed in only the liver and kidney (summarised in Table 7.9). Essentially, all lesions were of mild-moderate severity. The presence of these lesions was insufficient to explain why the animals died. However, it is possible that the animals did not survive long enough for significant changes to be demonstrated at the level of light microscopy.

Acute renal tubular necrosis and isolated parenchymal cell necrosis were observed only in animals of 24 months and older. The levels of transaminases in serum samples suggested more extensive damage to hepatocytes than was observed by microscopy.

TABLE 7.9 PATHOLOGICAL LESIONS AND SERUM TRANSAMINASES INDUCED BY THE INTRAVENOUS INJECTION OF E.COLI O26:B6 ENDOTOXIN (2 or 4 mg/100g bw) INTO FEMALE BN/BiRij RATS OF DIFFERENT AGES

Parameter	Frequency	
age	3 & 6 months	24 & 36 months
total number of animals	17	16
Major Liver Lesions		
Portal tract haemorrhage	2	6
Focal areas of parenchymal cell necrosis	5	7
Isolated parenchymal cell necrosis	0	6
Elevated serum transaminases	+ (5/16)	+++ (4/4)
Renal Lesions		
Hydronephrosis	0	16*
Renal tubular necrosis	0	10

+: mild (x 2 of normal)
+++: severe (x 8-20 of normal)
*: normal at this age

7.3.6 Serum Transaminases (experiment 6-10)

An elevation in both SGOT and SGPT is accepted as evidence of hepatocellular injury. Elevations in SGPT alone may be due to other factors, such as haemolysis. Serum transaminase elevations in female BN/BiRij rats injected with 2 or 4 mg of endotoxin per 100 g body weight were alluded to in the previous section. The results of the remainder of the serum transaminase estimations are summarised in Table 7.10. Elevated transaminases were not seen in six month old BN/BiRij or WAG/Rij rats injected with 1 mg per 100 g body weight of endotoxin or in 6 or 24 month old, female BN/BiRij rats injected with 0.1 µg per 100 g body weight of endotoxin and these two groups do not appear in the table.

Elevations in transaminases appear to be related to non-survival. Most non-survivors had marked elevations while survivors had only modest or no elevation. The role of hepatocellular injury in the progression of the animals to death is not clear.

7.3.7 Blood Glucose (experiment 5-10)

Blood glucose changes were observed in male BN/BiRij rats (aged 6 and 24 months), female BN/BiRij rats (aged 18 months) and female WAG/Rij rats (aged 6 and 24 months). Within any one age group there was no strain-related difference in the basal concentration of blood glucose. Twenty four month old rats had a slightly lower (but significantly so; $p < 0.05$) basal blood glucose concentration than did six month old animals. The mean blood glucose for the 24 month old rats was 5.82 mmol/l (standard deviation 0.70) and for the 6 month old rats was 6.41 (standard deviation 0.54). This difference was statistically significant ($p < 0.05$). Animals that died always had a blood glucose concentration of 1.5 mmol/l or less at the time of death. Some of the "non-survivors" had blood glucose concentration as low as 0.1mmol/l. No significant change in blood glucose was detected in rats injected with 0.1 µg/100 g body weight of endotoxin (experiment 10).

7.3.8 "Temperature Clamp" (experiment 5)

The prevention of heat loss in 24 month old BN/BiRij female rats had a marked effect upon survival. Only two of the animals died within the time course that we regard as characteristic of 24 month old animals of this strain. One of the animals that survived to 48 hours appeared ill and would probably not have survived if the experiment had been continued. The body temperature data is summarised in Figure 7.10. When the animals were removed from the cabinet for a period of one hour and maintained at an ambient temperature of 21°C, they experienced a rapid fall in body temperature which was reversed on return to the cabinet.

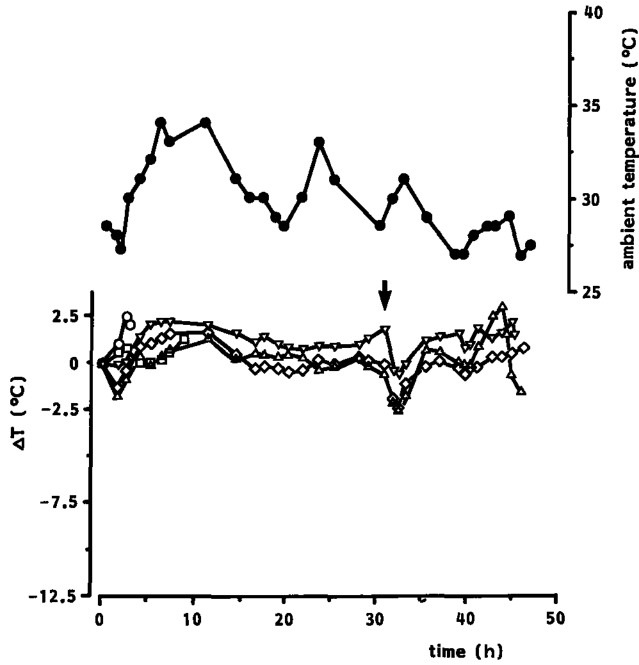


Fig. 7.10 Changes in body temperature in a group of female BN/BiRij rats (n=5) injected with E.coli O26:B6 endotoxin and maintained in a thermostatically controlled cabinet. (Arrow indicates removal from the cabinet for 1 hour).

TABLE 7.10 SUMMARY OF SERUM TRANSAMINASE MEASUREMENTS IN RATS OF DIFFERENT AGE, SEX AND STRAIN INJECTED WITH E.COLI 026:B6 ENDOTOXIN

Strain	Animal number	E.T.dose (mg/100 g bw)	Age (mo)	Sex	SGPT	SGOT	Survival
BN/BiRij	1	1	6	F	215	472	S
	2	1	6	F	24	151	S
	3	1	6	F	68	272	S
	4	1	6	F	839	1771	S
	5	1	6	F	24	130	S
BN/BiRij	2	10	6	F	1898	1471	NS
	3	10	6	F	18	18	NS
BN/BiRij	2	1	18	F	135	25	S
	3	1	18	F	1091	892	NS
	4	1	18	F	424	171	NS
	5	1	18	F	56	22	S
BN/BiRij	1	1	24	F	617	1062	NS
	2	1	24	F	782	1066	NS
	3	1	24	F	943	1578	NS
	5	1	24	F	31	2850	NS
BN/BiRij	1	1	24	M	90	380	NS
	2	1	24	M	151	410	NS
	3	1	24	M	503	643	NS
	4	1	24	M	238	356	NS
	5	1	24	M	141	513	NS
WAG/Rij	3	1	24	F	1290	978	NS
	4	1	24	F	1310	1685	NS
	5	1	24	F	698	526	NS

E.T.: endotoxin;
M: male;
F: female;
S: survivor; NS: non-survivor
normal SGPT: less than 100 iu/l
normal SGOT: less than 150 iu/l
bw: body weight

The two animals that died showed evidence of hepatocellular injury with elevated transaminases, as did the survivor that appeared ill at the end of the experiment. Both non-survivors had low blood sugar concentrations at the time of death (1.5 and 0.5 mmol/l respectively). The ill-looking survivor had a blood glucose concentration of 0.4 mmol/l at the time of sacrifice. The other two animals had a fall in blood glucose concentration from the basal level, of about 2.5 mmol/l by five hours after injection and this level was maintained until the end to the experiment when the animals were sacrified.

7.3.9 Endotoxin Absorption

"Endogeneous" endotoxin was detected with the LAL test in the portal blood of all animals but not in the systemic blood. Clearly, this endotoxin could have originated from the isolated loop but could equally well have come from anywhere along the length of the gut. Absorption of ^{51}Cr-labeled endotoxin from the isolated loops of jejunum and colon was measured directly by radioactivity counting and did not differ significantly as a function of age (Fig. 7.11).

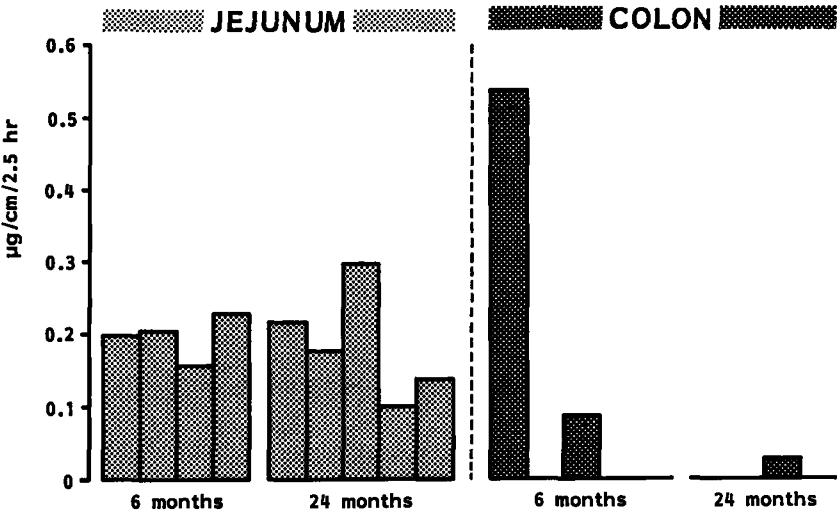


Fig. 7.11 Absorption of ^{51}Cr-labeled endotoxin from jejunal loops (containing 1 mg of endotoxin) and colonic loops (containing 0.5 mg of endotoxin) in situ.

7.4 DISCUSSION

Perhaps the most clear result from the series of experiments reported above is the dramatic difference between the sensitivity of young and aging rats to the lethal effects of endotoxin. All animals aged 24 months and older died following the intravenous injection of 1, 2 or 4 mg of endotoxin per 100 g body weight. Within this dose range, groups of animals given higher doses experienced an earlier onset and shorter duration of lethality. Eighteen month old rats given the 1 mg dose experienced only 40% mortality at the 48 hour endpoint. Mortality curves for male and female BN/BiRij rats were essentially the same. Although 24 month old female WAG/Rij rats also experienced 100% mortality over 48 hours, the time course was somewhat more prolonged.

An age-related increase in sensitivty to the lethal effects of endotoxins has been reported in mice. For example, Habicht (1981) reported that approximately 65% of 24 month old BALB/c and C57BL/6J mice were killed by the intravenous injection of 50 µg of E.coli O111:B4 endotoxin (phenol extract). This dose approximates to 125 µg/100 g body weight. Mortality was 13-17% in 14 month old animals and zero in 3-4 month old animals. Similar results were obtained by Hoffman-Goetz and Keir (1985) using 50 µg of endotoxin per 100 g body weight in C57BL/6 mice. Mortality was 57% in old animals and 10% in young animals (assessed over 4 days). Neither of these studies gave details about times of onset and duration of lethality in the various groups.

In the studies reported here, the age-related increase in sensitivity to endotoxin-induced lethality is loosely associated with (but not necessarily caused by) a prolongation of the plasma half life of endotoxin (determined both by ^{51}Cr-labeled endotoxin and by the LAL test). One problem with using ^{51}Cr as a label is that the site of labeling is unknown (G. Ramadori & K-H Meyer zum Buschenfelde; letter, Gastroenterology 83 521, 1982). It was suggested that much of the label may bind protein contaminants (which have a x 30 higher affinity for ^{51}Cr). In answer, Praaning- van Dalen et al. calculated that even if this were so, at least 78% of the ^{51}Cr would be bound to lipopolysaccharide in this preparation (D.P. Praaning-van Dalen, A. Brouwer, D.L. Knook; letter, Gastroenterology 83, 521, 1982). Another possible complication is the degradation of endotoxin with release of label (a possible problem with all radioactive labels). It is therefore important to realise that when ^{51}Cr chromate salts are injected intravenously, there is no age-related prolongation of half life (G.J.M.J. Horbach, personal communication).

The prolonged clearance reported here may reflect an age-related decline in reticuloendothelial capacity (reviewed by Brouwer & Knook, 1983). It is, however, known that prior exposure to endotoxin can induce a defect in the ability of human monocytes to bind, take up and degrade endotoxin (Larsen & Sullivan, 1985). Such prior exposure has been suggested as a possible explanation for a number of

age-related phenomena (Winchurch et al., 1982; Horan & Fox, 1984; Stith & McCallum, 1985). The source of this "endogenous" endotoxin has been suggested to be the gut. Evidence is also presented in Section 7.1.9 of this chapter that the composition of the gut flora may modulate sensitivity to the lethal effects of endotoxins. It is therefore important to note that no age-related increase in endotoxin absorption has yet been demonstrated (Section 7.3.9).

One of the major associations of lethality in the present series of experiments is a profound and progressive hypothermia. Early on, both survivors and non-survivors appeared to attempt to generate heat (by shivering) and to conserve heat by lowering the surface area to volume ratio and by pilo-erection. Over the first 60-90 minutes of the experiments, all animals experienced a modest fall in body temperature followed by a return towards normal. This tendency continued in survivors, which usually achieved a slight pyrexia. Non-survivors displayed a second phase of falling temperature which continued until death. At this time, the presumed behavioural attempts to generate and conserve heat were no longer observed. This pattern of response was seen in both young and old non-survivors. Young and old animals given a non-lethal dose of endotoxin (0.1 μg/ 100 g body weight) experienced no early fall in body temperature and within an hour of endotoxin injection, developed fevers of similar magnitude. This suggests that 24 month old animals of this strain do not have an overtly defective thermostatic mechanism. It is, however, possible that the larger doses of endotoxin could induce a defect. Prior exposure to endotoxin may modify the temperature response to endotoxins. Levenson (1978) reported that germ-free rats sustained a body temperature which was about 1°C lower than their conventional counterparts. This was associated with a lower basal metabolic rate (by about 20%) in the germ-free rats. Furthermore, germ-free guinea pigs develop fever when injected with endotoxin in the dose range 2-4 mg/Kg body weight. Conventionally kept guinea pigs become hypothermic within the same dose range (Abernathy & Landy, 1967). Germ-free guinea pigs also developed hypothermia when the dose was increased to 6 mg/Kg body weight.

Mechanical injury can produce a change in temperature regulation with an alteration in the threshold in the hypothalamus (the site of the "thermostat") for both heat production and heat loss. The result of this is that animals kept at an ambient temperature lower than thermoneutrality tend to lose heat. Many of the organism's responses to endotoxin are very similar to those observed after other forms of injury and probably form part of a stereotyped response. If endotoxin does induce such a change in hypothalamic thresholds, older animals seem to be much more sensitive to this effect than younger ones.

Ageing in humans is undoubtedly associated with impairment of thermo-regulatory mechanisms (Collins et al., 1982). In general, old people are less able to compensate for heat loss in cold environments. This is partly due to poor vasomotor control and an attenuated metabolic response to a cold stress (non-

shivering thermogenesis). The perception of changes in ambient temperature are also impaired. In mice, the ability to regulate body temperature has been linked to survival (Reynolds et al., 1985). As a group, older C57BL/6J mice display much greater variation in body temperature over time at an ambient temperature of 22°C than do young animals of the same strain. Older animals which managed to sustain their basal body temperature and showed least variation tended to survive longer. Death was always preceded by a progressive fall in body temperature.

Responses to temperature stresses in old (19-20 months old) C57BL/6J mice are also different from those in younger animals (Hoffman-Goetz & Keir, 1984). At an ambient temperature of 15°C, 6-7 months old animals were able to prevent a fall in body temperature. Animals aged 19-20 months showed profound falls in body temperature and even animals aged 13-14 months displayed some fall. At thermo-neutral conditions (30°C), 19-20 month old animals had significantly lower temperatures than did the younger animals. If a humidity stress was superimposed, the 13-14 month old animals also experienced a fall in body temperature. It would appear that the abnormality which underlies altered thermoregulation with age commences well before old age, since it can be uncovered by superimposing the additional stress of high humidity.

Changes in central (brain) pathways of thermoregulation may be one important age-related change, but changes in the ability to mount an appropriate metabolic response may also be important. In small animals, heat production is a much more important mechanism in thermostasis than is regulation of heat loss (Little, 1985). The reverse is true for large animals. Behavioural responses seem to be of equal importance in both groups. Kiang-Ulrich and Horvath (1985) examined the responses of male F344 rats of different ages to a prolonged cold stress of 5°C. The median survival age for this strain is 24 months. Animals aged 3 and 12 months had increases in food intake of more than 100% and maintained their body weight. Twenty one month old animals ate even more than the 12 month olds but were unable to maintain body weight. Twenty five month old rats experienced severe loss of body weight and showed only 46% survival at three weeks. It was suggested that the two older age groups were unable to generate metabolic heat as efficiently as did the younger animals.

Old animals may not only have disordered thermoregulation but may also have a defective ability to generate fever. Fever is defined as an increase in body temperature due to a re-setting of the hypothalamic thermostat at a higher level. It must be clearly distinguished from hyperpyrexia. Endotoxin has been a useful tool in elucidating the mechanisms of fever. Hort and Penfold (1911, 1912) showed that all "injection fevers" were the result of a single factor, namely contamination of the distilled water used as diluent. Filtering and autoclaving did not remove the pyrogenic factor. Siebert (1923) and Bourne and Siebert (1925) showed that the pyrogen was a product of gram negative bacteria (endotoxin). Most of the subsequent research has been performed in rabbits (and to some extent in

humans). The rabbit pyrogen test is still used as a means of detecting endotoxins. The lowest fever-inducing dose of endotoxin produces a brief fever within 45-90 minutes. With larger doses, the fever response becomes biphasic. Male NZW rabbits over two years of age have been shown to lose this second peak. Responses in humans are always monophasic (Dinarello, 1983). Humans are very sensitive to the pyrogenic effect of endotoxin with a minimum pyrexia-inducing dose in the range 0.1-0.5 ng/Kg body weight. There is is a latent interval of 90-120 minutes during which the subjects feel cold (due to the re-setting of the hypothalamic thermostat). The fever peak occurs at about 3-4 hours, with defervescence by 7 hours. Rhesus monkeys require a 10^3-10^4 times larger dose to induce fever. Responses in rodents are variable (Bodel & Miller, 1976).

A wide range of substances other than endotoxin are capable of causing fever and therefore researchers sought a common mediater. Atkins and Wood (1955) described a heat-labile substance circulating in experimental fevers and called it endogenous pyrogen (EP). It was shown to be different to endotoxin since it is heat-labile and tolerance does not develop with its repeated administration (as happens with endotoxin). EP is believed to be identical to interleukin 1 (IL 1). Interleukin 1 refers to a family of monokines which have very similar properties (Ihrie & Wood, 1985). Interleukin 1 production by cerebral astrocytes has also been demonstrated (Fontana et al., 1984). Interleukin 1 may be regarded as a hormone which plays a critical role in the orchestration of the acute phase response (Dinarello, 1983).

Endogenous pyrogen has no direct thermal action (Cooper et al., 1964). When it is given to patients with cervical cord lesions at the sixth cervical level, shivering is only seen in muscles whose innervation derives from segments above this level. The pre-optic area of the anterior hypothalamus is the only area which is sensitive to the pyrogenic effect of EP (Jackson, 1967). When EP is applied to this area, the latency before fever is only five minutes. Arachidonic acid metabolites are also implicated in the fever response (especially prostaglandin E_2), though they may not be essential. Some inhibitors abort the fever produced by prostaglandin E_2 but not that induced by EP (Cranson et al., 1976). Other hormones and neurotransmittors may also be important. For example, Brattleburo rats (which have hereditary diabetes insipidus and do not produce vasopressin) produce no fever response to endotoxin, though they do generate fever when arachidonic acid, prostaglandin E_2, prostacyclin, nor-adrenaline, morphine, or β-endorphin are injected into the cerebral ventricles. The pyrexial response to endotoxin is restored by co-administration of arginine vasopressin (Kandasamy & Williams, 1983).

It is unclear as to whether Interleukin 1/EP production is impaired with age. Studies using human peripheral blood monocytes indicate no impairment with age (Jones et al., 1984). Studies in mice either show no change with age or a diminished response (see Inamizu et al., 1985). The assay systems used in these

studies varied, and this may explain some of the conflicting results. It is also possible that Interleukin(s) 1 production is differentially affected by age. Clearly, there remains much scope for research in this field.

The discussion so far has intimated that changes in the temperature regulatory mechanism and the ability to produce "metabolic heat" may be implicated in the hypothermic response of old animals at a dose that produced no such response in young animals. Furthermore, by analogy with mechanical trauma, endotoxin may alter hypothalamic thresholds for heat loss and heat production. Prevention of heat loss has a markedly beneficial effect upon both survival and upon progression of hypoglycaemia (Section 7.3.8). Perhaps the application of external heat has a sparing effect upon metabolism so that the metabolic consequences of endotoxaemia are better tolerated. Hoffman-Goetz & Keir (1985) described a similarly beneficial influence on survival after endotoxin challenge by maintaining the mice at an ambient temperature of 30°C.

The precise pathogenesis of the hypoglycaemia is unknown. Some of the possible mechanisms are discussed in Section 7.1.7a. Hypothermia may itself have profound effects on carbohydrate and fat metabolism (Stoner et al., 1983). The possible effects of concentrating endotoxin in the adrenal gland are unclear (Mathison & Ulevitch, 1985). In old mice, the capacity for gluconeogenesis is impaired (Stith & McCallum, 1984). Glucocortocoid regulation of the enzyme PEP-CK (a rate limiting step in gluconeogenesis) is also impaired. Glucocorticoid binding to receptors on hepatocytes is increased in old age but PEP-CK levels are normal. Following endotoxin administration, PEP-CK activity may actually fall.

Gluconeogenesis is primarily a function of peri-portal hepatocytes. It is therefore of interest that the density of Kupffer cells is greatest in the peri-portal region and these Kupffer cells also have a higher phagocytic index (at least for latex particles) than do pericentral Kupffer cells (Sleyster & Knook, 1982). Since endotoxin is concentrated in Kupffer cells, it is tempting to speculate that hepatocytes in the peri-portal region might therefore be more likely to be influenced by mediated endotoxin effects. This hypothesis is worthy of further investigation.

Histological examination did not help to elucidate the cause of the increased sensitivity to endotoxin in older animals. Histological lesions were very mild or absent in animals that survived. In the non-survivors, the major change in the liver was focal necrosis and isolated parenchymal cell necrosis. This is consistent with studies on endotoxaemia in dogs (Nordstoga & Aasen, 1979) and E.coli bacteraemia in rats (Sato et al., 1982). The pathogenesis of the hepatocellular necrosis is unclear, though macrophage lysosomal enzymes (Tanner et al., 1983) and leukotrienes (Keppler et al., 1985) have been implicated.

The other major histological lesion was renal tubular necrosis. This may simply accompany decreased arterial pressure generally. Nonetheless, renal blood flow was normal or increased in dogs infused with endotoxin, even when the aortic pressure was low (Levy et al., 1983). This was believed to be mediated by such

mediators as histamine, kinins and prostaglandins. Renal blood flow began to fall after around 1½ hours of infusion. Although it was not demonstrated in this experiment, leukotrienes are very potent constrictors of the renal artery (Section 7.1.7b).

In conclusion, older rats are dramatically more sensitive to the lethal actions of endotoxins than are younger ones. These non-survivors displayed progressive hypothermia and hypoglycaemia. Age-related changes in the capacity of the systems responsible for in temperature regulation and metabolism may sensitise animals to an endotoxin challenge at a dose than can be tolerated by their young counterparts, even though these systems may not be overtly abnormal in un-stressed conditions.

CHAPTER VIII

FINAL DISCUSSION AND SUMMARY

Endotoxins are high molecular weight complexes which form an integral part of the gram negative bacterial cell wall. The term "endotoxin" was first introduced and defined by Richard Pfeiffer in 1892. Nevertheless, it was Peter Ludwig Panum (a Danish pathologist) who must be credited with their discovery and the description of their basic pathophysiological properties. The innate sensitivity of higher organisms to endotoxins varies greatly. For example, baboons are some 10^4-10^5 times more resistant than humans. Within a given species (mice) endotoxin sensitivity has been shown to be governed by a single autosomal gene on chromosome 4 (LPS gene). Sensitivity to endotoxic effects may also change as an organism progresses through its lifespan and this issue is addressed in Chapter VII.

Latterly, great strides have been made in understanding the chemical structure of endotoxins. They comprise a long polysaccharide moiety which is covalently bound to a lipid moiety - so called lipid A (hence the term, lipopolysaccharide or LPS). Lipid A is composed of beta 1-6-linked D-glucosamine disaccharide units which carry a variety of amide- and ester-linked fatty acids as well as pyrophosphate groups. Most of the biological properties of endotoxins are concentrated in the lipid A. It has been further determined that the amide-linked fatty acids and the 1-phosphate group are critical determinants of biological activity. Studies from Japan on synthetic lipid A analogues strongly suggest that there are multiple active sites.

Numerous methods have been described for the detection of endotoxins but most of these are too cumbersome to apply to clinical practice. There is only one test available at present which can be so applied - the Limulus amoebocyte lysate (LAL) test. This test is based on the observation that endotoxins can activate the coagulation system of the horseshoe crab (Limulus polyphemus). Lyophilised preparations of these clotting proteins are now available and can be combined with the use of chromogenic substrates to produce a very sensitive quantitative test for endotoxins. Despite problems with plasma proteins interfering with the test, sensitivity is no longer disputed. What still remains to be established is to what extent LAL activation predicts <u>other</u> biological properties. For example, it has been shown that some synthetic lipid A analogues are fully active in their effects on the immune system but may fail to activate LAL (Chapter I, section 1.1.4). Notwithstanding, no better test is currently available and this test has been used in all the studies reported in this monograph.

Interactions with various cells and plasma mediator systems have been described, but for the purposes of this monograph, interactions with cells of the lympho-reticular system are the most important. The major immunological effects of endotoxins are summarised in Table 8.1.

TABLE 8.1 IMMUNOLOGICAL EFFECTS OF LPS

1. B cell mitogenicity
2. Polyclonal antibody synthesis
3. Adjuvanticity - enhancement
   - suppression
4. Immunogenicity
5. Regulation of mucosal immunity
6. Macrophage activation

Of these effects, only regulation of mucosal immunity has been shown to be relevant under physiological conditions (Section 1.2.9). Germfree (GF) mice immunised orally with the TI-1 (thymic independent, type 1) antigen TNP-LPS showed good splenic PFC responses in the absence of added T cells. Addition of T cells from conventionally-maintained mice of the same strain suppressed this response and T cells from GF mice had no effect. Furthermore, prolonged oral administration of the TD (thymic dependent) antigen, sheep red blood cells (SRBC) results in unresponsiveness to a systemic challenge with the same antigen (oral tolerance) in conventionally-maintained animals. This phenomenon of oral tolerance is not seen in GF mice or in conventionally-maintained mice of the (LPS unresponsive) C3H/HeJ strain. It was later shown that this oral tolerance arises from the generation of large numbers of suppressor T cells in the Peyer's patches of conventionally-maintained endotoxin-sensitive mouse strains. This is evidence that gut-derived endotoxin exerts a negative-regulatory effect on the gut-associated lymphoid tissue (GALT) under basal conditions. It is therefore possible that endotoxins may induce other immunological effects under non-basal conditions. This issue is addressed in Chapters IV-VI.

In Chapter I, the important notion is raised that many endotoxic effects are not direct but are mediated by cells of the mononuclear phagocyte system and their secretory products. The list of secretory products of mononuclear phagocytes (Table 1.3) is ever increasing. The concept that discrete sub-populations of mononuclear phagocytes respond differently to LPS (Chapter I, Table 1.4) is also introduced. Lastly, prior exposure to LPS can exert a potent effect on mediator production (Chapter I, Table 1.5). Perhaps the most important group of mononuclear phagocytes (in any discussion on endotoxin) are the hepatic Kupffer cells. These cells are responsible for the removal of the bulk of the LPS that gains access to body fluids. These cells function not only as phagocytic cells, but can also function as antigen presenting cells in immune responses. Evidence was

recently presented at the 1985 Kupffer Cell Symposium that post-phagocytic Kupffer cells can (and do) leave the sinusoids and migrate to regional lymph nodes (Hardonk, 1985).

Systemic endotoxaemia can occur in a variety of liver diseases in which Kupffer cell function is believed to be aberrant. Furthermore, immunological changes (polyclonal elevations of serum immunoglobulins, circulating autoantibodies) occur which are very reminiscent of the changes which may be produced experimentally with endotoxins (Table 8.1). Unpublished results from studies performed in Manchester on patients with obstructive jaundice reveal that "spontaneously active" circulating blood lymphocytes increase in number by a factor of 4-5.

The method used for the detection and characterisation of these "spontaneously active" cells was developed specially for these studies. After separation of peripheral blood mononuclear cells (after the method of Bøyum, 1968), T cell surface antigens were detected with the monoclonal antibodies OKT_3, OKT_4 and OKT_8 (Ortho Diagnostic Systems Ltd.). A peroxidase-labeled second antibody was used to identify these cells in cytocentrifuge preparations. Since the cells had been previously incubated with ^{3}H-thymidine for two hours, autoradiography allowed the identification of "spontaneously active" cells. "Spontaneously active" cells could also be detected with liquid scintillation counting (which compared very favourably with autoradiography: Chapter III, Figure 3.2).

The major problem with the technique for the identification of the "spontaneously active" cells by means of their surface antigens was that it was very cumbersome and it was only practical to count a relatively small number of cells. A better approach to this problem might be to first separate cells into the desired populations by means of a fluorescence-activated cell sorter (FACS). A variety of immunological investigations, including enumeration of "spontaneously active" cells with their attempted characterisation, were performed in patients with bronchiectasis, in patients following moderate/major surgery and in elderly subjects in order to assess whether endotoxins might be relevant in the genesis of the immunological changes associated with these conditions.

Bronchiectasis (Chapter IV) is characterised by pathological dilatation of the airways with an impairment of the ability to clear airways secretions. Most of the subjects studied produced copious amounts of purulent sputum. The most striking immunological changes in this group of patients was a high frequency of polyclonal elevations of circulating immunoglobulins, together with circulating autoantibodies in low titre. Low levels of endotoxin were detected in the peripheral blood of some of these subjects but in none of the controls. Gram negative bacteria were isolated from the sputum of 75% of the subjects and were observed on gram staining in 95%. The mitogenic responses to phytohaemagglutin, concanavalin A and pokeweed mitogen were slightly (but significantly) depressed. This was associated with a slight (but significant) excess of lymphocytes bearing the surface antigens associated with cytotoxic/suppressor T cells. Most subjects had an excess of

"spontaneously active" peripheral blood lymphocytes (Chapter IV, Fig. 4.5). It is suggested that the gram negative bacteria which colonise the ectatic airways are responsible for the generation of the immunological abnormalities and possibly also for the airflow obstruction (by inducing the production of leukotrienes by polymorphonuclear leukocytes). A role for endotoxin in the genesis of secondary amyloid is also suggested.

Evidence is presented in Chapter VI that endotoxaemia is not infrequent after moderate/major surgery. This was demonstrated both for humans and for rhesus monkeys. The most likely source for this endotoxin is the gastrointestinal tract. Spillover of endotoxin into the systemic circulation can be explained by a transient impairment of reticuloendothelial function associated with enhanced absorption of endotoxin from the gut. Anaesthetic agents may be directly toxic to Kupffer cells as well as produce changes in splanchnic blood flow. The trauma of surgery itself may also reduce splanchnic blood flow as well as induce blockade of reticuloendothelial (RES) clearance function, perhaps as a result of circulating tissue debris complexed to fibronectin. Whatever the precise mechanism, depressed RES activity is well documented following many kinds of traumatic injury. Measurement of plasma fibronectin has been claimed to be a useful indicator of RES activity (Blumenstock et al., 1979). The time course for the depressed fibronectin levels observed in this study when compared with what is known of the time course of post-operative RES depression suggests that any relationship is indirect. Furthermore, it is reported in Chapter V that plasma fibronectin concentration tends to increase as a function of age, and old age is associated with diminished RES activity (Brouwer & Knook, 1982).

All of the post-operative patients had a marked excess of "spontaneously active" lymphocytes by the sixth post-operative day. Limited data on the characterisation of these cells suggest that they are mainly cytotoxic/suppressor T cells. Endotoxaemia was demonstrated in 16-26% of the human subjects within the first three post-operative days. This suggests that either endotoxin absorption is enhanced, endotoxin handling is impaired, or both. It is suggested that endotoxin exposure could be responsible for the lymphocyte activation. Even though endotoxaemia was demonstrated in only a minority of the subjects, it is known that endotoxin can produce immunological changes in humans at concentrations below the threshold of the LAL test (Chapter I, section 1.2.7). In this regard, it is also important to note that endotoxins can maximally stimulate human monocytes to interleukin 1 production at a concentration as low as 100 pg/ml (Chapter I, section 1.3.4.2). It is also believed that the hepatic Kupffer cells are the main source of IL-1 in the human body (Chapter I, section 1.3.4.2).

Numerous age-related changes have been described in both humans and experimental animals. These changes tend to be more marked in cellular immunity than in humoral immunity. The most obvious morphological change is thymic involution. Many immunogerontologists have made the unwarranted assertion that

this change underlies an age-related immunodeficiency disorder. This entity appears nowhere in the International Classification of Diseases and no functional sequelae in terms of susceptibility to infectious agents has ever been established. The notion that old age is associated with an increased susceptibility to infectious disease is largely based on uncontrolled clinical observations. It cannot be too strongly stated that the acquisition of accurate data on the incidence of specific infections in elderly people is notoriously difficult. This difficulty was recently highlighted by Ellis et al. (1985) who reported on admissions to a regional infection unit in Aberdeen (Scotland) which operated an "open door" policy. Only 30% of the patients over the age of 70 who were sent to the unit with a diagnosis of an infection were actually proved to have one. The major pathogens encountered in clinical geriatric practice gain entry to the host through a mucosal surface. The early events in the process of invasion do not involve specific immunity at all. Polymorphonuclear leukocytes and non-specific factors in blood and secretions are of critical importance during this phase. No consistent abnormalities in these parameters have been described (Horan et al., 1985). Furthermore, mucosal-associated immunity is minimally affected by age (Wade & Szewczuk, 1984). What is not disputed is that underlying disease may predispose to infection (Chandra, 1983) or that infection may have more serious consequences in old age (Fox, 1984). Even studies of experimental infections in mice (the species from which most of the unwarranted conclusions were drawn) do not give consistent results (Gardner & Remington, 1977; Patel, 1981; Emmerling et al., 1979; Matsumoto et al., 1979). Lovik and North (1985) report that the LD_{50} of Listeria monocytogeneses was 2-4 times greater for old (19-30 months) AB6F mice than for young adults of the same strain. When equivalent doses were given, no difference was found between young and old animals in their ability to generate specific antibody or to generate splenic T-cells capable of adoptively immunising young recipients against a lethal challenge of Listeria monocytogenes. Furthermore, the level of memory immunity to reinfection on days 28 and 117 after an immunising challenge was comparable between young and old animals. It is the author's belief that investigations into the thermostatic and metabolic responses are much more likely to give useful insights into how an aged individual deals with an infective challenge.

One age-related immunological change that is emphasised in this monograph is the increase in incidence of autoantibodies with advancing age. Mechanisms of autoantibody productions are discussed (Chapter V, section 5.3.3) and the possibility of polyclonal B cell activation is highlighted. A number of authors have considered the possibility that endogenous exposure to endotoxin could be responsible for the production of autoantibodies (Cohen & Ziff, 1977; Winchurch et al., 1982; Wade & Szewczuk, 1984). In the study reported in this monograph, "spontaneous" lymphocyte activation was seen in a sizeable minority of the subjects studied. Systemic endotoxaemia was recorded in five of these subjects. It is suggested that the two phenomena may be related. Nevertheless, several subjects

had evidence of considerable lymphocyte activation without any evidence of endotoxaemia. It must be recalled that endotoxin can enhance the mixed lymphocyte reaction at a concentration that cannot be measured (Chapter I, section 1.2.7) and that human monocytes are maximally stimulated to IL-1 production at very low endotoxin concentrations (Chapter I, section 1.3.4.2).

The above considerations on endotoxins and immune ageing prompted a series of experiments which were designed to investigate endotoxin handling in old age. The most dramatic finding was the markedly increased sensitivity to the lethal effects of endotoxin observed in old rats (36 months) when compared to young adults (6 months). Indeed, the increased sensitivity could be observed in 18 month old animals and was fully developed by 24 months. Most experimental gerontologists accept the criteria presented in Chapter V for the selection of appropriate animals for ageing research. In brief, an animal must derive from a species or strain which displays an approximately rectangular survival curve, which displays an age related increase in the incidence of multiple pathological lesions and that the animal must have passed the median survival age for that species or strain. The reasons for these criteria are competently addressed in the publication of Zurcher et al. (1982). It is clear that neither 24 mont old BN/BiRij rats nor 24 month old WAG/Rij rats have reached this median survial age (Chapter VII, Fig. 7.4). This raises an important question about the selection of animals for ageing reseach. Application of the above criteria may be crucial (at present) when the nature of the ageing process itself is the relevant issue. This is not necessarily so when one is interested in age-related phenomena. Ageing does not magically begin at the median survival age as some investigators seem to believe.

This age-related sensitivity to endotoxin-induced lethality is associated with a prolongation of the half life of endotoxin in the circulation which barely reaches statistical significance. It is, in itself, unlikely to explain the increased sensitivity. Perhaps clues may be taken from other associations. All non-survivors developed profound hypothermia and ample evidence is presented in Section 7.4 that thermostatic mechanisms are impaired with age, and that such impairment may also be observed in animals that are not aged according to the above definition. Rodents are much more dependent on production of heat than on prevention of heat loss for maintaining their body temperature (Little, 1985). This heat generation is mainly metabolic and the demands placed upon the metabolic reserve must compete with the demands placed by the endotoxin itself. If the reserve capacity of an ageing organism's metabolism becomes progressively contracted, this could explain the age-related increase in sensitivity. That metabolic efficiency is attenuated with advancing age is supported by the observations of Kiang-Ulrich and Horvath (1985). These authors clearly demonstrated that although old animals increased food intake in response to a prolonged cold stress (Chapter VII, section 7.4) they also displayed progressive weight loss.

The major metabolic change seen in non-survivors in the experiments reported here is a progressive and severe hypoglycaemia. This may in part relate to the hypothermia and in part to the inability to induce the enzyme, PEP-CK (a rate limiting step in gluconeogenesis). Whatever the cause, prevention of hypothermia by applying external heat ("temperature clamp") had a markedly beneficial effect upon survival and was associated with a much smaller fall in blood glucose over the time course of the experiment.

Perhaps these observations provide a lesson for experimental gerontology. One fairly consistent finding throughout the gerontology literature is that when isolated parameters are studied, only modest age-related changes are found which are sometimes of doubtful significance. The experiments reported in this monograph tested one aspect of a very complex system - the integrated response to injury. Using death as an endpoint, the results are nothing short of dramatic. These results underline the assertion that a number of minor changes within a complex, integrated system might ultimately be manifested as a major change. The results reported have only indicated a direction for further assessing changes in the response to injury in old age. Nevertheless, they are convincing enough to make such investigation a worthwhile exercise.

> "Gerontologists must learn to put the molecular viewpoint back into a correct biological perspective, in which the systemic aspects of the organisms are seen to be, in some respects, more basic than the molecular".
>
> G.A. Sacher, 1968

HOOFDSTUK VIII

ALGEMENE DISCUSSIE EN SAMENVATTING

Endotoxinen zijn complexen met een hoog moleculair gewicht, welke een onderdeel vormen van de celwand van gram negatieve bacterien. De term "endotoxine" werd in 1892 geintroduceerd en gedefinieerd door Richard Pfeiffer. Het was echter Peter Ludwig Panum, een Deens patholoog, die het endotoxine ontdekte en zijn pathofysiologische eigenschappen beschreef. De aangeboren gevoeligheid van hogere organismen voor endotoxine varieert aanzienlijk. Bavianen b.v. zijn 10^4 tot 10^5 minder gevoelig dan mensen. Aangetoond werd dat bij de muis de gevoeligheid voor endotoxine gereguleerd wordt door een enkel autosomaal gen op chromosoom 4 (LPS gen). De gevoeligheid voor endotoxine kan echter veranderen wanneer een organisme veroudert. Dit onderwerp wordt behandeld in hoofdstuk VII.

De laatste tijd is grote vooruitgang geboekt bij het ophelderen van de chemische struktuur van het endotoxine. Endotoxine bestaat uit een lange polysaccharide keten, die covalent gebonden is aan een lipide, het zgn. lipide A; vandaar de term lipopolysaccharide (LPS). Lipide A is opgebouwd uit β,1-6, D-glucosamine disaccharide eenheden, waaraan d.m.v. amide- en esterverbindingen vrije vetzuren en pyrofosfaatgroepen gekoppeld zijn. De meeste biologische eigenschappen van het endotoxine zijn toe te schrijven aan het lipide A. De amidegebonden vetzuren en de 1-fosfaatgroep blijken de essentiële determinanten van de biologische activiteit te zijn. Studies aan synthetische lipide-analogen uitgevoerd in Japan, suggereren duidelijk dat er meerdere actieve centra zijn.

Er zijn veel methoden beschreven voor het aantonen van endotoxine, maar de meeste zijn te omslachtig om te worden toegepast in de kliniek. Alleen de Limulus Amoebocyte Lysate (LAL)-test kent dit bezwaar niet. De test is gebaseerd op het gegeven dat endotoxine het stollingssyteem van de degenkrab (Limulus polyphemus) kan activeren. Gevriesdroogde preparaten van deze stollingsfactoren zijn nu verkrijgbaar die, in combinatie met chromogene substraten, een gevoelige kwantitatieve test voor endotoxine blijken te zijn. De sensitiviteit van deze test wordt niet langer meer in twijfel getrokken, ondanks het feit dat plasma-eiwitten kunnen interfereren met de test. Wat nog verder onderzocht dient te worden is in hoeverre LAL-activatie een maat is voor andere biologische eigenschappen. Het is b.v. aangetoond dat sommige synthetische lipide A-analogen wel actief zijn in hun interactie met het immuunsysteem, maar niet in staat zijn tot LAL-activatie (Hoofdstuk I, sectie 1.1.4). Op dit moment is er echter geen betere test beschikbaar en de LAL-test is dan ook gebruikt in alle experimenten beschreven in dit proefschrift.

Er zijn interacties van endotoxinen met verschillende soorten cellen en plasma-gemedieerde systemen beschreven. In het kader van dit proefschrift echter zijn de interacties met de cellen van het lymforeticulaire systeem de belangrijkste. De voornaamste immunologische effecten van endotoxinen zijn samengevat in Tabel 8.1.

TABEL 8.1 IMMUNOLOGISCHE EFFECTEN VAN LPS

1) B cel mitogeen
2) Polyclonale antilichaamsynthese
3) Adjuvans - stimulerend
   - onderdrukkend
4) Immunogeen
5) Regulatie van de immuniteit van de mucosa
6) Macrofaagactivatie

Het fysiologische belang van deze effecten is echter alleen aangetoond voor de regulatie van de immuniteit van de mucosa (Hoofdstuk I, sectie 1.2.9). Kiemvrije muizen die oraal geïmmuniseerd werden met het thymus onafhankelijke type 1 antigeen TNP-LPS hadden een goede PFC-respons van de milt in de afwezigheid van toegevoegde T cellen. Toevoeging van T cellen, geisoleerd uit conventioneel gefokte muizen van dezelfde stam, onderdrukten deze respons, terwijl T cellen van kiemvrije muizen dit effect niet hadden. Bovendien bleek dat conventioneel gefokte muizen niet meer reageerden op een intraveneuze dosis van een thymus afhankelijk antigeen: rode bloedcellen van schapen (SRBC), na langdurige orale toediening van dit antigeen. Dit laatste fenomeen wordt orale tolerantie genoemd. Orale tolerantie wordt niet waargenomen in kiemvrije muizen of conventioneel gefokte muizen van de LPS-ongevoelige muizestam C3H/HeJ. Later werd aangetoond dat deze orale tolerantie veroorzaakt wordt door het ontstaan van groté aantallen T suppressorcellen in de Peyerse plaques van conventioneel gefokte, endotoxine gevoelige, muizenstammen. Dit bewijst dat endotoxine afkomstig uit het darmkanaal, onder normale omstandigheden, een negatief regulerend effect heeft op het darm-geassocieerde lymfoïde weefsel. Het is dan ook mogelijk dat endotoxine onder abnormale omstandigheden andere immunologische effecten heeft. Dit onderwerp wordt besproken in de Hoofdstukken IV-V.

In Hoofdstuk I wordt het belangrijke concept besproken dat vele effecten van het endotoxine niet direct zijn, maar uitgeoefend worden via de cellen van het mononucleaire fagocytensysteem en hun gesecreteerde producten. De lijst van producten die door mononucleaire fagocyten geproduceerd worden (Hoofdstuk I, Tabel 1.3) wordt nog steeds langer. Het idee dat verschillende subpopulaties van mononucleaire fagocyten verschillend reageren op LPS wordt eveneens geintroduceerd in dit hoofdstuk (Hoofdstuk I, Tabel 1.4). Als laatste blijkt dat eerdere blootstelling aan LPS een sterk effect kan hebben op de productie van mediatoren (Hoofdstuk I, Tabel 1.5). De belangrijkste groep van mononucleaire fagocyten,

voor zover het endotoxine betreft, zijn misschien de Kupffercellen van de lever. Deze cellen zijn verantwoordelijk voor het verwijderen van het meeste LPS dat opgenomen wordt in de lichaamsvloeistoffen. Bovendien hebben deze cellen niet alleen een fagocyterende, maar ook een antigeen presenterende functie bij immuunreacties. Op het Kupffer Cell Symposium in 1985 werd bewijs gepresenteerd dat Kupffercellen, na fagocytose, de sinusoïden kunnen verlaten en vervolgens naar de regionale lymfknopen migreren (Hardonk, 1985).

Endotoxemie kan voorkomen bij een verscheidenheid van leverziekten, waarbij wordt aangenomen dat de Kupffercellen een functionele afwijking vertonen. De immunologische veranderingen die daarbij optreden, zoals b.v. polyclonale verhoging van serum immunoglobulinen en circulerende autoantilichamen, doen sterk denken aan de veranderingen die experimenteel geinduceerd kunnen worden met endotoxinen (Tabel 8.1). Ongepubliceerde resultaten uit studies uitgevoerd in Manchester toonden aan dat in patienten met een obstructieve geelzucht het aantal "spontaan actieve" circulerende bloedlymfocyten verhoogd was met een factor 4-5.

De methode om "spontaan actieve" cellen te detecteren en te karakteriseren werd speciaal ontwikkeld voor deze studie. Na isolatie van perifere mononucleaire bloedcellen (volgens Bøyum, 1968) werden T cel oppervlakte antigenen aangetoond met de monoclonale antilichamen OKT3, OKT4 en, OKT8 (Ortho Diagnostic Systems Ltd., Engeland). Een tweede antilichaam gekoppeld aan peroxidase werd daarna gebruikt om deze cellen te identificeren in cytospin preparaten. De "spontaan actieve" cellen konden vervolgens met behulp van autoradiografie aangetoond worden, omdat ze tevoren gedurende twee uur met getritieerd thymidine geincubeerd waren. Deze "spontaan actieve" cellen konden ook gedetecteerd worden met behulp van liquid scintillation counting, een methode die minstens zo goed bleek als de autoradiografische (Hoofstuk III, Fig. 3.2).

Het belangrijkste probleem bij de identificatie van "spontaan actieve" cellen was dat de techniek die gebruik maakt van monoclonale antilichamen een omslachtige was; het was alleen practisch om een klein aantal cellen te tellen. Een betere benadering zou zijn om eerst de cellen te scheiden in de gewenste populaties met behulp van een fluorescentie-geactiveerde celsorteerder (FACS). Een verscheidenheid aan immunologische onderzoeken inclusief het tellen van "spontaan actieve" cellen met hun karakterisering werd verricht op materiaal van patienten met bronchiectasieën, patienten die een (middel)grote operatie ondergingen en oudere mensen. Op deze wijze werd bestudeerd of endotoxine belangrijk is voor het ontstaan van de immunologische veranderingen geassocieerd met deze condities.

Bronchiectasieën (Hoofstuk IV) worden gekarakteriseerd door een pathologische verwijding van de luchtwegen gecombineerd met een verminderd vermogen om het sputum te verwijderen. De meesten van de bestudeerde patienten produceerden grote hoeveelheden purulent sputum. De opvallendste immunologische verandering in deze groep patienten was het veelvuldig voorkomen van polyclonale verhoging van circulerende immunoglobulinen gecombineerd met circulerende auto-

antilichamen in een lage titer. Kleine hoeveelheden endotoxine werden waargenomen in het perifere bloed van sommige van deze patienten, maar niet in de controles. Gram negatieve bacteriën konden bij 75% van de patienten geïsoleerd worden uit het sputum. Bij 95% van de patienten kon de aanwezigheid van bacterien aangetoond worden met behulp van een gramkleuring. Bovendien bleek dat de mitogene respons op phytohaemagglutinine, concanavaline A en pokeweed mitogeen licht, maar significant onderdrukt was. Dit ging gepaard met een lichte, maar significante overmaat aan lymfocyten die oppervlakteantigenen droegen kenmerkend voor cytotoxische/suppressor T cellen. De meeste patienten hadden een overmaat aan "spontaan actieve" perifere bloedlymfocyten (Hoofdstuk IV, Fig. 4.5). Er wordt gesuggereerd dat gram negatieve bacterien die de verwijde luchtwegen koloniseren verantwoordelijk zijn voor het ontstaan van de immunologische afwijkingen en misschien ook voor de obstructie van de luchtstroom doordat de productie van de leukotrienen in polymorfonucleaire leucocyten wordt geinduceerd. Dat endotoxine een rol speelt bij het ontstaan van secundair amyloid wordt eveneens gesuggereerd.

In Hoofdstuk VI wordt bewijs geleverd dat bij onderzoek in de mens en de rhesusaap endotoxemia niet zeldzaam voorkomt na een (middel)grote chirurgische ingreep. De meest waarschijnlijke bron van dit endotoxine is het maagdarmkanaal. De aanwezigheid van endotoxine in de circulatie kan worden verklaard door een tijdelijke dysfunctie van het reticuloendotheliale systeem, die gepaard gaat met een verhoogde absorptie van endotoxine uit de darm. Anesthetica zouden een direct toxisch effect kunnen hebben op Kupffer cellen en/of veranderingen kunnen induceren in de splanchnische bloedstroom. Het trauma als gevolg van de operatie zelf zou eveneens de bloedstroom in de splanchnische vaten kunnen verminderen en/of een blokkade van de klaringsfunctie kunnen induceren, welke het gevolg kan zijn van circulerend weefseldebris dat gecomplexeerd is met fibronectine. Wat het exacte mechanisme ook moge zijn, verminderde RES-activiteit na verscheidene vormen van traumatische schade is goed gedocumenteerd. Plasma fibronectinespiegels worden beschouwd als een goede indicator van de RES-activiteit (Blumenstock et al., 1979). Wanneer de afname van de fribonectine spiegels, zoals die gemeten zijn in deze studies, vergeleken wordt met de afname die optreedt in post-operatieve RES-onderdrukking, dan wordt de suggestie gewekt dat enige correlatie een <u>indirecte</u> is. Bovendien blijkt uit de resultaten gepresenteerd in Hoofdstuk V dat de plasma fibronectine concentratie toe lijkt te nemen als functie van de leeftijd, terwijl hoge leeftijd juist geassocieerd is met een verminderde RES-activiteit (Brouwer & Knook, 1982).

Alle post-operatieve patienten hadden een duidelijke overmaat aan "spontaan actieve" lymfocyten op de zesde dag na de operatie. De beperkte gegevens verkregen uit de karakterisatie van deze cellen suggereren dat het voornamelijk cytotoxische/ suppressor T cellen zijn. Endotoxemia kan worden aangetoond in 16-26% van de patienten tijdens de eerste drie dagen na operatie. Dit suggereert

dat de absorptie van endotoxine verhoogd is, of dat de verwerking van endotoxine verlaagd is, of dat een combinatie van beide processen optreedt. Blootstelling aan endotoxine zou verantwoordelijk kunnen zijn voor de lymfocytenactivatie. Endotoxemia werd slechts aangetoond in een klein aantal van de gevallen. Het is echter bekend dat endotoxine immunologische veranderingen bij mensen teweeg kan brengen in concentraties die liggen onder de detectiegrens van de LAL test (Hoofdstuk I, sectie 1.2.7). In dit opzicht is het belangrijk om op te merken dat endotoxine in staat is humane lymfocyten te stimuleren tot interleukine 1 productie bij de lage concentratie van 100 pg/ml (Hoofdstuk I, sectie 1.3.4.2). Er wordt aangenomen dat met name de Kupffercellen van de lever de voornaamste producenten van het interleukine 1 zijn in het menselijk lichaam (Hoofdstuk I, sectie 1.3.4.2).

Talrijke leeftijdsafhankelijke immunologische veranderingen zijn beschreven in mensen en proefdieren. De veranderingen die optreden bij de cellulaire immuniteit lijken opvallender te zijn dan die in de humorale immuniteit. De opvallendste morfologische verandering echter is de involutie van de thymus. Veel immunogerontologen hebben de ongerechtvaardigde bewering gedaan dat deze verandering het gevolg zou zijn van een verouderingsgerelateerde immunodeficiëntie. Deze aandoening is niet opgenomen in de International Classification of Diseases en de gevolgen, zoals de gevoeligheid voor infectieziekten zijn niet beschreven. Het idee dat veroudering gepaard gaat met een toename in de gevoeligheid voor infecties is voor het grootste gedeelte gebaseerd op ongecontroleerde klinische observaties. Het kan niet genoeg benadrukt worden dat het verkrijgen van nauwkeurige gegevens over de incidentie van specifieke infectieziekten in oudere mensen een zeer moeilijke zaak is. Dit probleem werd recent door Ellis et al. (1985) benadrukt. Hij beschreef patienten die verwezen werden naar een regionale polikliniek in Aberdeen (Schotland), welke een "open deur" politiek volgde. Van de patienten die ouder dan 70 jaar waren en die verwezen werden naar de kliniek met de diagnose van een infectie, kon bij slechts 30% van de gevallen aangetoond worden dat ze ook werkelijk een infectie hadden. De belangrijkste pathogenen die men tegenkomt in de klinisch geriatrische praktijk dringen binnen via een van de slijmvliezen. De specifieke immuniteit speelt bij de eerste gebeurtenissen van het invasieproces geen enkele rol. In deze fase zijn polymorfonucleaire leucocyten, niet-specifieke factoren in het bloed en secretoire vloeistoffen van essentieel belang. Consistente afwijkingen konden in deze parameters niet gevonden worden (Horan et al., 1985). Bovendien blijkt dat veroudering de mucosa-geassocieerde immuniteit slechts in geringe mate beinvloedt (Wade & Szewczuk, 1984). Het is echter wel zo, dat een sluimerende ziekte kan predisponeren voor infectie (Chandra, 1983), of dat een infectie serieuzere consequenties heeft voor oudere mensen (Fox, 1984). Hoewel de studies naar experimenteel geinduceerde infecties in muizen geen consistente resultaten geven, waren deze de aanleiding voor de meeste van de ongefundeerde aannames (Gardner & Remington, 1977; Patel, 1981; Emmerling et al., 1979;

Matsumoto et al., 1979). Lovik en North (1985) beschreven dat de LD_{50} voor Listeria monocytogenes 2-4 maal zo groot was voor oude (19-30 maanden) AB6F muizen dan voor jonge volwassen muizen van dezelfde stam. Er werd geen verschil waårgenomen tussen oude en jonge dieren in het vermogen om specifieke antilichamen of T cellen te produceren, die in staat waren om jonge recipienten door adoptieve immunisatie te beschermen tegen een dodelijke dosis Listeria monocytogenes. Bovendien bleek dat het niveau van memory-immuniteit op dag 28 en 117 vergelijkbaar was voor oude en jonge dieren. De auteur is van mening dat onderzoeken naar thermostatische en metabole parameters waarschijnlijk eerder bruikbare inzichten zullen verschaffen in de wijze waarop een ouder individu reageert op een dreigende infectie.

Een van de immunologische veranderingen die wordt benadrukt in dit proefschrift, is de toename in het voorkomen van autoantilichamen bij veroudering. De mechanismen voor autoantilichaamproductie zijn bediscussieerd (Hoofdstuk V, sectie 5.3.3) en de mogelijkheid dat B cellen polyclonaal worden geactiveerd, is benadrukt. Een aantal auteurs hebben de mogelijkheid bediscussieerd dat endogene blootstelling aan endotoxinen verantwoordelijk zou kunnen zijn voor de productie van autoantilichamen (Cohen & Ziff, 1977; Winchurch et al., 1982; Wade & Szewczuk, 1984). Uit de alhier beschreven studie bleek dat "spontane" lymfocytenactivatie waargenomen werd in een klein aantal van de bestudeerde individuen. Endotoxemia kon worden aangetoond in vijf van deze patienten. Het is mogelijk dat de twee fenomenen gerelateerd zijn. Een aanzienlijke lymfocytenactiviteit werd echter aangetoond bij verscheidene patienten zonder aantoonbare endotoxemia. Men moet niet vergeten dat endotoxemia in ondetecteerbaar lage concentraties de mixed lymphocyte reaction kan verhogen (Hoofdstuk I, sectie 1.2.7) en dat endotoxine in zeer lage concentraties humane monocyten maximaal stimuleert tot interleukine 1 productie (Hoofdstuk I, sectie 1.3.4.2).

De bovengenoemde overwegingen over endotoxine en veroudering zijn de aanleiding geweest voor een serie experimenten waarin de verwerking van endotoxine tijdens veroudering bestudeerd werd. Het meest dramatische resultaat was de waarneming dat de gevoeligheid voor de lethale effecten van het endotoxine enorm verhoogd was in oude ratten (36 maanden) vergeleken met jonge ratten (6 maanden). De toegenomen gevoeligheid kon reeds waargenomen worden bij 18 maanden oude ratten en had zijn hoogste waarde al bereikt bij 24 maanden oude ratten. De meeste experimentele gerontologen accepteren de kriteria voor de selectie van de juiste proefdieren voor verouderingsonderzoek zoals beschreven in Hoofdstuk V. Kort samengevat moet een proefdier behoren tot een soort of stam die een bij benadering rechthoekige overlevingscurve vertoont. Verder moet de incidentie van multipele pathologische lesies met veroudering toenemen en moet het proefdier de mediane leeftijd van de soort of stam overschreden hebben. De redenen voor deze kriteria zijn op adequate wijze beschreven in de publicatie van Zurcher et al. (1982). Het is duidelijk dat noch de 24 maanden oude BN/BiRij

ratten, noch de 24 maanden oude WAG/Rij ratten deze mediane leeftijd bereikt hebben (Hoofdstuk VII, Fig. 7.4). Dit werpt een belangrijke vraag op over de selectie van proefdieren voor verouderingsonderzoek. Toepassing van bovengenoemde kriteria zou cruciaal kunnen zijn, indien het verouderingsproces zelf onderzocht wordt. Dit hoeft echter niet het geval te zijn indien men geinteresseerd is in verouderingsgerelateerde verschijnselen. Veroudering begint niet als bij toverslag wanneer de mediane leeftijd is bereikt, zoals sommige onderzoekers schijnen te geloven.

De toegenomen gevoeligheid voor lethale doses endotoxine tijdens veroudering gaat gepaard met een verlenging van de halfwaardetijd van endotoxine, die nauwelijks statistisch significant is. Deze verlengde halfwaardetijd alleen is waarschijnlijk niet voldoende om de verhoogde gevoeligheid te verklaren. Misschien kunnen er verklaringen gevonden worden in andere waarnemingen. Alle ratten die dood gingen ontwikkelden een verregaande hypothermie. In Hoofdstuk 7, sectie 7.4 wordt aangetoond dat de temperatuurregulatie afneemt tijdens veroudering. Deze verminderde regulatie kan ook worden waargenomen in dieren die niet oud zijn volgens de bovengenoemde definities. Knaagdieren zijn voor het constant houden van hun lichaamstemperatuur sterk afhankelijk van hun warmteproductie (Little, 1985). Deze warmteproductie is voornamelijk het gevolg van metabole processen en stelt dus eisen aan de metabole reserve, welke moeten concurreren met de eisen die endotoxine-verwerking stelt. Als de metabole capaciteit van een verouderend organisme afneemt, zou dat de toegenomen endotoxine gevoeligheid bij veroudering kunnen verklaren. De afname van de metabole efficiëntie bij veroudering wordt ondersteund door de observaties van Kiang-Ulrich en Horvath (1985). Deze auteurs toonden duidelijk aan dat oude dieren, ondanks de toegenomen voedselopname als reactie op een voortdurende koude-stress (Hoofdstuk VII, sectie 7.4), toch een progressief verlopend gewichtsverlies lieten zien.

De voornaamste metabole verandering die waargenomen werd bij de overleden ratten in de alhier beschreven experimenten was een progressieve en ernstige hypoglycemia. Dit kan voor een deel samenhangen met de hypothermie en voor een deel met het onvermogen om het enzym fosfo-enol pyruvaat carboxykinase (dit enzym is snelheidsbeperkend in de gluconeogenese) te induceren. Wat de oorzaak ook moge zijn, overleving werd bevorderd door de dieren in een warme omgeving te plaatsen, zodat hypothermie werd voorkomen. Bovendien resulteerde deze preventie in een veel kleinere daling van de glucosespiegel.

Misschien zijn deze waarnemingen een les vòor de experimentele gerontologie. Een veelvuldig voorkomend fenomeen in de gehele gerontologische literatuur is dat wanneer geïsoleerde parameters worden bestudeerd, er slechts bescheiden verouderingsgerelateerde veranderingen worden waargenomen, welke soms een twijfelachtige betekenis hebben. De experimenten beschreven in dit proefschrift betreffen één aspect van een zeer gecompliceerd systeem: de geïntegreerde reactie op letsel. De resultaten lijken nogal dramatisch, omdat de dood als eindpunt is gebruikt. De

resultaten ondersteunen de bewering dat een aantal kleine veranderingen in een complex geïntegreerd systeem uiteindelijk kunnen leiden tot een grote verandering. De beschreven resultaten geven alleen een richting aan voor het vaststellen van de veranderingen die plaatsvinden tijdens veroudering als reactie op letsel. Dit neemt niet weg dat de resultaten overtuigend genoeg zijn om verder onderzoek een waardevolle onderneming te maken.

> "Gerontologists must learn to put the molecular viewpoint back into a correct biological perspective, in which the systemic aspects of the organisms are seen to be, in some respects, more basic than the molecular".
>
> G.A. Sacher, 1968

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